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Contributions of Nucleotide and Base Excision Repair to the Repair of DNA Oxidative Base Damage in *Saccharomyces cerevisiae*

by

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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In The Name Of God, The Most Beneficent, The Most Merciful

To My Parents

With all my love

Without your endless love and sacrifice this work would certainly never be possible. You have been great sources of inspiration, love, support, encouragement, strength and all that was needed for a work well achieved throughout my whole life. I owe my deepest thanks and appreciation to you. Thanks for always being there for me, for always giving and wanting nothing in return, for being so patient with me right through the completion of this work. Thanks for loving me generously much to let me go oceans away but supporting me so closely.

In return of your sacrifices too vast to be appreciated enough, I have so little to give; and that is my heartfelt appreciation and thanks, and to say that I love you and I am so much proud of you.

DECLARATION

This work has not been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed... ... ...

Abstract

Oxidative DNA damage has been implicated in the aetiology of cancer, ageing and a broad range of diseases. Oxidative base lesions are thought to be removed primarily by the base excision repair pathway (BER) with nucleotide excision repair (NER) removing bulky helix distorting lesions. However, recent evidence in *E.coli* and humans has implicated NER in the repair of oxidative base damages. Such evidence is not well documented in yeast. In order to further investigate the contribution of NER to the repair of oxidative base lesions in *Saccharomyces cerevisiae,* a series of mutant strains were constructed that were deficient in NER, or in both NER and BER by disruption of the *RAD14* gene via transformation in the wild type and *OGG1* deficient strains respectively. In addition, another set of mutants was created by transforming the cells with a high copy number plasmid that over-expresses the *OGG1* gene. The frequency of spontaneous forward mutation to canavanine resistance (Can^R) was determined in these strains. The *ogg1* mutant, the NER defective *rad14*, and the *ogg1rad14* double mutant all showed similar significant increases in the frequency of spontaneous mutation to Can^R relative to the wild type. The over-expression of the *OGG1* gene caused a complete suppression of the frequency of spontaneous mutation to Can^R in the *ogg1* strain, no suppression in the *rad14*, and a slight suppression in the *ogg1rad14* mutant. These results suggested that NER also has a role in the removal of oxidative base damages on its own or possibly via a DNA-protein or a protein-protein interaction between the BER and NER enzymes. In the canavanine forward mutation assay, the nature and position of the lesions in the gene are not known and this limits our ability to interpret the results. A more specific approach was undertaken by studying the role of NER in removal of 8-oxoG by determining the frequency of spontaneous GC to TA mutations using a specific tester system, which detects a GC to TA base substitution event as a Cyc^+ reversion mutation . These experiments did not show a significant increase in the frequency of Cyc^+ reversion in the *rad14* single mutant relative to the wild type, but there was a significant increase in reversion to Cyc^+ in the *ogg1* and *ogg1rad14,* with the latter showing an additional increase in Cyc^+ reversion relative to the single *ogg1* mutant. This suggested that the principal mechanism that removes 8-oxoG lesion from DNA is BER but NER has also a role, which becomes significant under conditions where BER is inactive or where the BER repair proteins are saturated due to the presence of increased damage. Finally, a method was applied to detect oxidative DNA damage at nucleotide resolution in the *MFA2* locus of *S.cerevisiae.*

Abbreviations

6-4PP	Pyrimidine (6-4) Pyrimidone photoproduct
8-oxoG	8-oxo-7,8-dihydrodeoxyguanine
A	Adenine
ADP	Adenosine diphophate
AMP	Adenosine monophosphate
AP	Apurinic/Apyrimidinic
APE	AP endonuclease
ATP	Adenosine Triphosphate
BER	Base Excision Repair
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CHO	Chinese Hamster ovary (cell line)
CPD	Cyclobutane Pyrimidine Dimer
CS	Cockayne Syndrome
dATP	2'-deoxyadenosine triphosphate
ddNTP	2',3'-dideoxynucleoside triphosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
dRpase	Deoxyribophosphodiesterase
ds	Double Stranded
DTT	Dithioerythreitol
EDTA	Ethylendiamine-tetraacetic Acid
Endo III	Endonuclease III
ERCC	Excision Repair Cross Complementing
FAPY	Formamidopyrimidine
FPG	Formamidopyrimidine Glycosylase
FpgSS	Fpg sensitive site
G	Guanine
HNPCC	Hereditary Nonpolyposis Colon Cancer
HPLC	High Performance Liquid Chromatography
kb	Kilo base pairs
kDa	Kilodalton
LET	Linear energy transfer
LMPCR	ligase mediated PCR

MB	Methylene blue
MPC	Magnetic particle concentrator
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NEF	Nucleotide Excision Factor
NER	Nucleotide Excision Repair
NMR	Nuclear Magnetic resonance
NTS	Nontranscribed Strand
OGG1	Oxyguanine Glycosylase I
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMS	Post Meiotic Segragation
Pol	Polymerase
r.p.m	Round per minute
R.T	Room temperature
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPA	Replication Factor/protein A
SDS	Sodium dodecyl/lauryl sulphate
SSB	Single strand break
ssDNA	Single stranded DNA
T	Thymine
TAE	Tris acetate EDTA
TBE	Tris Boric EDTA
TCR	Transcription Coupled Repair
TFIIH	Transcription Factor II H
Tg	Thymine glycol
TRCF	Transcription Repair Coupling Factor
Tris	Tris(Hydroxymethyl) Aminoethane
tRNA	Transfer RNA
TS	Transcribed strand
TTD	Trichothiodystrophy
UV	Ultraviolet
XP	Xeroderma Pigmentosum
YAC	Yeast artificial chromosome

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Chapter 1

General Introduction

The carrier of genetic information in all living organisms, DNA, is continually subjected to undesired structural changes considered as "DNA damage". Radiations such as UV light and X-rays and numerous exogenous or endogenous chemical agents can damage DNA in many different ways. Apart from the DNA damaging agents present in the environment or produced inside the cells, errors during DNA replication, recombination, or repair processes, and the inherent instability of the chemical bonds in DNA, are the other main factors responsible for the alterations occurring to the genetic material (Friedberg *et al.,* 1995). In order to safeguard the integrity of the genome against the constant threats and prevent the hazardous consequences of DNA damage, all living organisms have been equipped with a complex network of repair systems to continuously repair the DNA damages (Hoeijmakers, 1993; Braithwaite *et al.*, 1999).

DNA damage and repair play a fundamental role in health and disease. DNA damage interrupts vital processes such as replication and transcription and may give rise to chromosomal aberrations, mutations and cell killing. Accumulation of unprepared or misrepaired DNA damage has been considered a critical initiating event in carcinogenesis (Bohr *et al.,*1989). Many cancer prone human hereditary diseases such as Xeroderma pigmentosum are directly related to a deficiency in DNA repair

mechanisms (Bohr, 1989; Woods, 1998). DNA damage and repair has also been considered to be the underlying event in the ageing process (Kirkwood, 1989; Wei_YH, 1998).

With so many fascinating vital biological phenomena related to DNA damage and repair, it is not surprising that there has been great interest in this subject over the past few decades. Although large areas of our knowledge about DNA damage and repair mechanisms still remain in the dark, intensive research to understand these processes provides a clear way ahead.

The work presented in this thesis, is a study of the repair of oxidative base damage in DNA in the simple eukaryote, *Saccharomyces cerevisiae*. It is appropriate to discuss the reasons why this organisms has been considered as an ideal model in numerous studies of DNA damage and repair before starting the main subject.

1.1Yeast as a model eukaryote

The yeast *Saccharomyces cerevisiae* is probably the most ideal eukaryotic micro-organism for biological research. Some of the properties that make yeast a suitable model include: rapid growth, dispersed cells, the ease of replica plating and mutant isolation. Furthermore, yeast is non-pathogenic and cheap to culture.

S.cerevisiae can grow mitotically in both the haploid and diploid states. This important property facilitates convenient isolation of recessive mutations using the haploid state, and genetic complementation tests using its diploid state. Yeast strains

are viable with numerous genetic markers, and a highly versatile DNA transformation system, makes yeast extremely suitable for genetic engineering or gene cloning applications. Unlike most other organisms, integrative recombination of transforming DNA in yeast takes place exclusively by homologous recombination. Therefore, foreign DNA with at least a partially homologous fragment can be integrated at specific locations in the yeast genome. Hence, wild type genes can be conveniently replaced by genetically engineered or disrupted alleles. These techniques have had a significant contribution in elucidating the roles of great number of genes and proteins *in vivo*. Yeast cells can also be transformed by synthetic oligonucleotides, as integrating or non-integrating plasmids can be easily introduced into yeast cells. This has facilitated the production of numerous altered forms of proteins. Also the identification of structural genes from almost any genetic trait can be undertaken by complementation from plasmid libraries. More advanced techniques such as two hybrid screening system for studying protein–protein interactions and the use of YACS in cloning large fragments of DNA are invaluable for studies of other organisms (Sherman, 1998, see html-1).

During the last 20 years, increasing number of biologists have used yeast as a model system. As a result, numerous aspects of molecular and cell biology of yeast have been studied in great detail. One of the most significant outcomes of this was the sequencing of the whole *S.cerevisiae* genome completed in 1996 (Dujon, 1996). One area of cell and molecular biology, which has benefited enormously from the advantages of yeast as a model system, is DNA damage and repair. *Saccharomyces cerevisiae* has been employed as a model eukaryotic system to study DNA repair

since the isolation of yeast mutants sensitive to killing by UV radiation (Nakai &Matsumoto, 1967).

Apart from the general advantages of yeast discussed above, the striking homology between the DNA repair proteins and genes to those in humans, and the availability of a large number of disruption and point mutants for genes with role in DNA repair, has made yeast an attractive model for DNA repair scientists.

The Yeast genome

S.cerevisiae has a haploid set of 16 chromosomes ranging from 200 to 2,200 kb. The entire yeast chromosomal DNA, comprising 12, 052 kb, has been sequenced. A total of 6, 183 open reading frames (ORF) of above 100 amino acid long protein sequences have been reported. It is predicted that 5,800 of these ORF correspond to actual protein coding genes and a large number of ORF correspond to shorter proteins. Unlike most other organisms, yeast has a highly compact genome with genes representing 72% of the total sequence. The average size of a yeast gene is 1.45 kb. Ribosomal RNA is coded by an estimated 120 copies of a single tandem array located on chromosome XII, and 262 tRNA genes have been identified.

In addition, most laboratory yeast strains have approximately 30 movable DNA elements or retrotransposons. Mitochondrial DNA encoding components of the mitochondrial translational machinery and 15% of the mitochondrial proteins, is another nucleic acid entity in yeast cells. The 2 μm circle plasmids with the sole function of their own replication, ds RNA viruses and circular single stranded RNA are present in most strains and can also be considered as part of the yeast genome (Sherman, 1998; see html-1).

Growth and life cycle of the yeast *Saccharomyces cerevisiae*

The vegetative cell division takes place by budding, which involves the formation of an outgrowth from the mother cell, followed by nuclear division, formation of a cell wall, and cell separation. Haploid and diploid cells have some distinct morphological differences. Generally diploid cells are larger and have buds that appear at opposite poles while haploid cells form adjacent buds. The age of the mother cell can be determined by the number of bud scars on it's cell wall and each mother cell dies after production of usually 20-30 buds. Normally the laboratory haploid strains have a doubling time of about 90 minutes, reaching a maximum density of 2×10^8 cells/ml in complete YPD medium.

S.cerevisiae can be maintained as heterothallic or homothallic strains. The life cycles of heterothallic and homothallic strains of *S.cerevisiae* are shown in Fig.1.1. Under nutrient deficiency, both strains sporulate. Sporulation process involves meiosis of the diploid cell and production of four progeny haploid cells encapsulated as spores (or ascospores) in a sac like structure called an ascus. Each ascus contains two *MATa* and two *MATα* haploid cells. Exposure to nutrient conditions, makes the spores germinate and mating of *MATa* and *MATα* can take place. However, mechanical separation of the spores by micromanipulation enables the haplophase of the heterothallic strains to be isolated and stably maintained. From these, haploid strains can be provided. However, in homothallic strains, the haploid cells are capable of mating type switching which makes the *MATa* cell produce *MATα* buds and vice versa. Therefore homothallic strains have only a transient haplophase state (Sherman, 1998, see html-1).

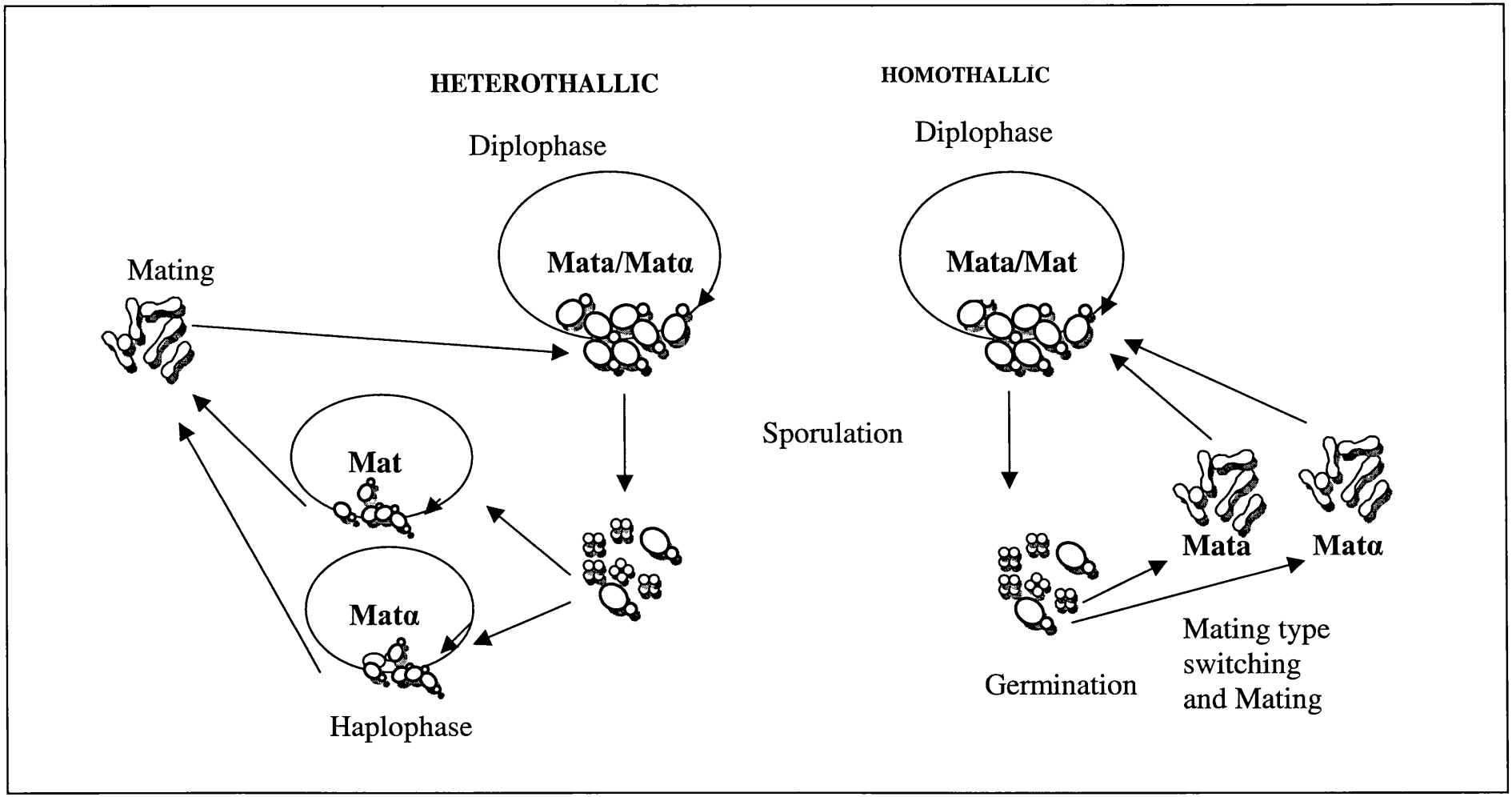


Figure 1.1. Life cycles of the heterothallic and homothallic strains of *S.cerevisiae*. (*Adapted from Sherman., 1998, see html-1*).

1.2 DNA damage

1.2.1 Environmental DNA damaging agents

1.2.1.1 UV radiation

Ultra violet light is a component of the range of wavelengths produced by the Sun. Only a small amount of UV light reaches the earth's surface because most of it is either absorbed by the ozone layer or reflected back into the space. The wavelengths from about 790 nm (red) to 430 nm (violet) are contained within the range visible to the human eye. Ultra violet light is the light with wavelengths shorter than the human eye can see and it is subdivided into three wavelength bands. UVA (315-400nm), UVB(290-315nm) and UVC(220-290nm).

UVA radiation, is involved in production of photochemical smog and also in fading and causing damage to plastic, fabrics and paints. UVB, constitutes only 1% of the solar radiation and most of it is absorbed by the ozone. Nevertheless, it is the most biologically significant band because of its ability to cause damage to molecules such as DNA. UVB effects plants and aquatic organisms by reducing their growth rate. Increased exposure to UVB results in skin cancer and damage to the eye tissue. Despite its harmful effects, UVB has some biological benefits. These include a role in production of vitamin D in humans. UVC is another component of UV radiation that can cause damage at biological level and has the same damaging potential as UVB. Nevertheless, it is not as biologically significant; as it is completely absorbed by the ozone and other gases, not finding it's way to the earth surface. Since the absorption

peak for DNA is located within the wavelengths of 254-260 nm, 254 nm UVC is used as a model mutagen.

Atmospheric ozone is a strong absorber of the harmful UV radiation but like other natural resources, its integrity is threatened by modern life. The amount of ozone shielding the earth from the dangerous effects of UV radiation has decreased by almost 3% globally over the last decade, which is responsible for increasing incidence of skin cancer in human population (UV radiation, 1998, see html-2).

1.2.1.1.1 DNA damage by UV radiation

Cyclobutane pyrimidine dimers

The principal and the best studied lesion induced by UV light at 260nm is the cyclobutane pyrimidine dimer (CPD), which is created by the formation of a covalent link between adjacent pyrimidines in the same DNA strand. As a result of this covalent link, the 5,6 double bond of the both pyrimidine bases become saturated and a four membered structure is formed (Fig. 1.2a) (Setlow and Carrier, 1966). This bulky lesion constitutes 70-85% of the total DNA damages produced by UV (Mitchel and Nairn, 1989).

There is evidence indicating that CPDs cause significant distortion in the DNA double helix. The amount of distortion in the DNA structure as a result of the presence of a cis-syn dimer has not been clearly determined but separate studies on DNA structure have demonstrated bendings as much as 7^{o} (Wang and Taylor, 1991) and 27^{o} (Van Houten *et al*, 1986; Husain, 1988) (Fig. 1.3) in the double helix as a result of the

presence of CPDs.

The formation of cyclobutane pyrimidine dimers in DNA was believed to be a random process for many years. However, recent findings suggest that the yield of cyclobutane pyrimidine dimers in naked DNA can be influenced by factors such as nucleotide composition, sequence context and the nature of the nucleotides flanking potential dimer sites. (For a detailed review see Friedberg *et al.*, 1995)

The pyrimidine-pyrimidone (6-4) photoproduct

Pyrimidine-pyrimidone photoproducts (6-4PPs) are the second most common DNA lesion constituting 15-30% of DNA damages induced by UV light at 260nm (Fig. 1.2b). 6-4PPs are noncyclic adducts and result from the formation of a covalent bond between C6 and C4 of two adjacent pyrimidines in the same DNA strand (Mitchell and Nairn, 1989). 6-4 PP is also considered as a bulky lesion and it introduces a major distortion in the double helix (Taylor *et al.*, 1988).

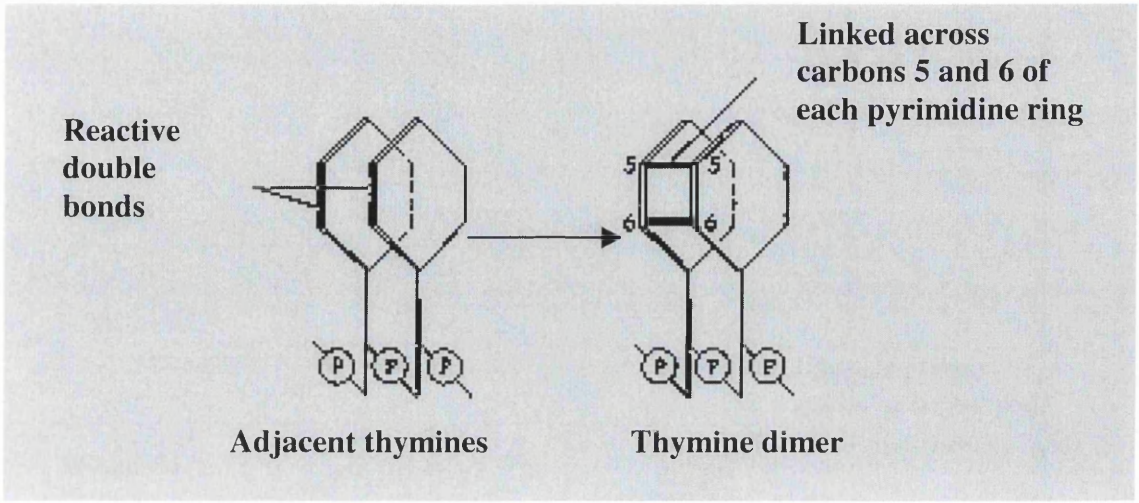
Other photoproducts in DNA

UV radiation can induce other types of lesions at lower frequencies. These include pyrimidine monoadducts, purine dimers and pyrimidine hydrates. Thymine glycol, which is one of the major forms of base damage induced by ionising radiation, can also be produced in DNA by UV. Single or double strand breaks in DNA, DNA-protein and DNA-DNA cross-links are other types of lesions produced occasionally by UV light (Friedberg *et al.*, 1995).

Indirect DNA damage by UV radiation –photosensitization

The lesions discussed above, result from the direct absorption of photons by DNA

a)



b)

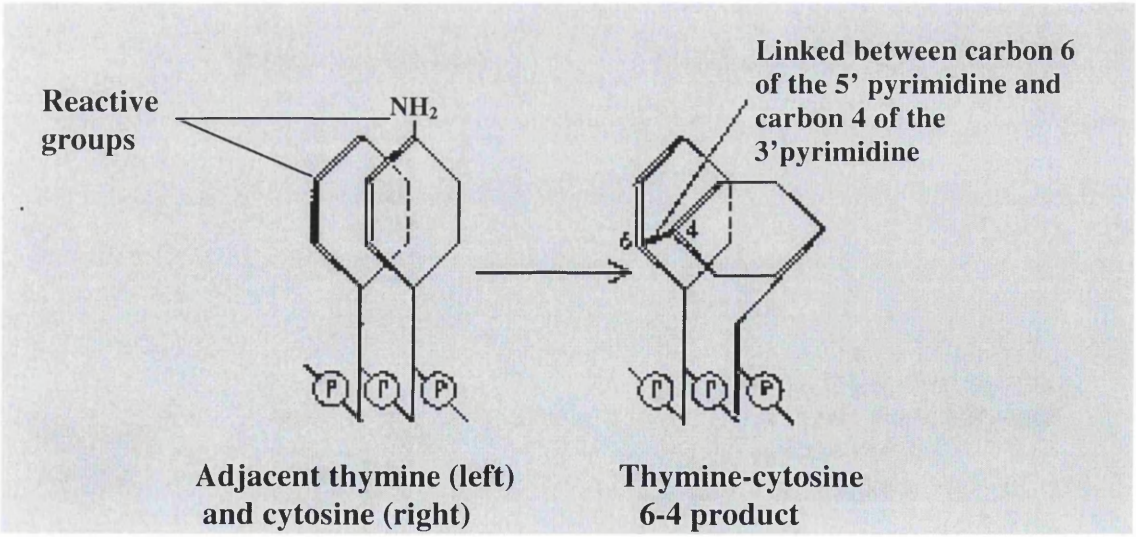


Figure 1. 2 Two important pyrimidine dimer photoproducts produced by ultraviolet light. (*Adapted from Watson et al., 1988*).

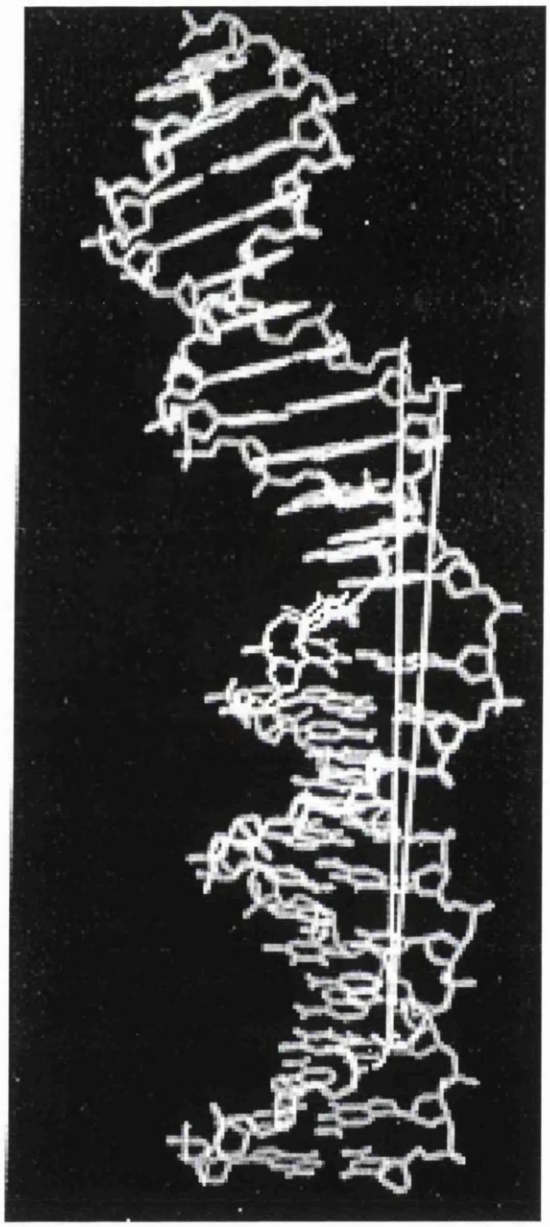


Figure. 1.3. Energy minimized structure for duplex DNA containing a CPD. The structure has been oriented to display a helical kink of 27° and unwinding of 19.7° associated with the presence of dimer. (*Adapted from Friedberg et al., 1995*)

bases. DNA damage by UV can also be introduced in an indirect way, where the energy of UV radiation is absorbed by other molecules (sensitizer molecules) which can then transfer this energy to DNA and cause damage. This phenomenon is known as photosensitization (Lamola, 1974). Photosensitizers include exogenous drugs such as 8-methoxyosoralen and endogenous agents such as riboflavins, aromatic amino acids and various ketones such as acetophenone. Some photosensitizers such as acetophenone, can induce the formation of thymine dimers at longer wavelengths of UV light, without activating the photoreversal repair reactions. At these longer wavelengths, the formation of other adducts such as 6-4 products are avoided. Thus these photosensitizers can serve as extremely useful damage inducing agents in the study of the repair of thymine dimers (Lamola, 1969; Umlas *et al.,* 1985).

The energy of UV light can also be transferred to oxygen resulting in the formation of highly reactive oxygen species which can then react with DNA and cause oxidative DNA damage (Piette *et al.,* 1986).

1.2.1.2 Chemical agents that damage DNA

Historically, the potential of chemicals as lethal weapons, motivated the early research on DNA damage by chemical agents (Brookes, 1990). However, based on the fact that DNA damage can interrupt replication in rapidly dividing cancer cells and therefore preventing the progress of the disease, chemicals that induce DNA damage have also been employed in medicine to fight against cancer. Cancer chemotherapy has inspired intense research into the mechanisms of DNA damage by chemicals. Increased public awareness of mutagens and carcinogens present in the

environment, has also provoked a demand for research on chemically induced DNA damage. There are numerous DNA damaging chemicals with a variety of mechanisms to introduce the damage. Some classes of these chemicals are well characterised and have been used as model mutagens in DNA damage and repair research. These include:

Alkylating agents

These are a diverse group of electrophilic compounds ranging from potent mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to methyl methane sulfonate (MMS) with less mutagenic potential. Alkylating agents react with nucleophilic centres in DNA and result in addition of alkyl groups. Alkylating agents target numerous sites in DNA. These sites are mainly the nitrogen or oxygen atoms of DNA bases (Singer and Kusmierek, 1982). An example of the damages induced by alkylating agents is O6-alkylguanine, which is a miscoding lesion and results in GC to AT transitions (Loveless, 1966; Brookes, 1990).

Base analogues

Analogous bases, with different base pairing properties, can be incorporated into DNA during replication giving rise to base substitution mutations. Among the base analogues, the halogenated uracil derivatives, 5-bromouracil, 5-fluorouracil and 5-iodouracil have been well studied. These thymine analogues, when present in replicating DNA, give rise to transition mutations (Freese, 1959).

Crosslinking agents

These are a group of compounds that can induce the formation of intrastrand and

interstrand cross links in DNA. Formaldehyde, nitrous acid, mitomycin C, nitrogen and sulphur mustards, some platinum derivatives and photoactivated psoralens are examples of cross linking chemicals (Friedberg *et al.,* 1995). Interstrand cross links can completely block DNA replication and transcription by preventing strand separation. Therefore, cross linking agents such as cis-diamminedichloroplatinum(II) (cisplatin) have been employed extensively as anti tumour drugs in cancer chemotherapy (Pinto and Lippard, 1985).

Intercalating agents

These chemicals, intercalate between the native bases to cause perturbation within the DNA structure. The presence of these lesions during DNA replication can give rise to the insertion or deletion of bases leading to frameshift mutations. The acridine mustards belong to this category and have been well studied (Ames and Whitfield, 1966).

1.2.1.3 Ionising radiation

These are radiations with high enough energy to remove tightly bound electrons from their orbits making the atoms charged or ionised. This kind of radiation is produced as a result of the decay of an unstable atom releasing energy as ionising radiation in the form of particles or waves which are in fact bundles of energy (photons). The most common types of radiation include alpha, beta and positron particles, gamma and x-rays, and neutrons (Basics of radiation and radioactivity, see html-2).

Background or naturally occurring ionising radiation

Life started in the presence of an ever-existing background of ionising radiation. The natural sources of ionising radiation include the radioactive materials such as uranium and thorium present in the air, in rocks and soil, in the water in the oceans and even in our body. One of the well known naturally occurring radioactivities in our environment is radon gas, which is produced as a result of the decay of uranium. Being an inert gas, radon itself is not harmful because, before any chance of decay, we breathe it in and out. It is the decay products of radon gas in the form of particles that are hazardous. These can be deposited in our lungs and by decaying give us a radioactive dose. Radon gas has been suggested to be the second largest cause of lung cancer by the USA Environmental Protection Agency.

Cosmic rays are another source of naturally occurring ionising radiation. A large percentage of this, comes from outside of the solar system and some is released from our sun during solar flares. These radiations consist of very high energy particles or high energy photons and mouns. However, only a very small amount of them penetrate the earth's surface. The largest portion reacts with the atmosphere, producing lower energy radiations in different forms such as photons, electrons and neutrons. Examples of naturally occurring elements made by cosmic ray interactions are Carbon 14 and Potassium 40, which are created by cosmic ray interactions primarily with the atmospheric gases nitrogen and oxygen. The atmosphere and the earth's magnetic fields act as shields against cosmic radiations (Radioactivity in nature, Radiation and us, see html-2; Eisenbud and Gesell, 1998).

Man made sources of ionising radiation

Ionising Radiation have been used for over a hundred years by humans. One of the first examples of an ionising radiation used in vast areas of human life is X-rays. These were discovered by Roentgen in 1895. X-rays are produced whenever high energy electrons strike a heavy metal target like tungsten or copper. When the electrons hit the heavy metal some of them reach the nucleus of the metal atom where they are deflected. The deflection causes an energy loss in electrons which is released as X-rays. X-rays are employed in medicine in modern techniques such as angiography, breast imaging, computed tomography (CAT scans), magnetic resonance imaging (MRI), radiotherapy and commonly used diagnostic X-rays to produce simple images of the bones and tissues in the body. Apart from medical exposure we can be exposed to very small amount of X-rays emitted from our television sets or smoke detectors (Radiation and us, Medical sources of Radiation, see html-2).

Radioactive isotopes are being used in wide range of areas such as scientific research, medicine and warfare. Nuclear medicine employs radioactive isotopes to create images that show the function of organs by introducing small amount of radioactive material into the body. Radioactive isotopes are also used in cancer radiotherapy.

Nuclear power reactors and the fission products produced from nuclear weapon detonation are other man made sources of exposure to ionising radiation.

Surprisingly, it has been estimated that the man made sources of radiation make up only 18% of the average annual dose to the public, 82% being the natural sources of

radiation (Medical sources of radiation, Radiation and us, see html-2; Eisenbud and Gesell, 1998)

Effects of ionising radiation

At doses similar to what our body receives every day as background radiation, cells can repair most of the damage rapidly, but at higher doses, unrepaired damage can result in mutation or cell death. Mutated cells may divide and give rise to cancer. Hence, radiation exposure can increase the cancer risk. At even higher radiation doses, extensive damage to the cells result in a failure of tissues and organs to function normally, a condition which gives rise to radiation sickness or death. (Radiation and risk, see html-2)

DNA damage by ionising radiation

When ionising radiation hits living cells, it deposits energy into molecules such as DNA. Linear energy transfer (LET), described as the average energy loss due to collisions per distance travelled by a particle is a unit to quantify the energy deposition by ionising radiation (Goodhead, 1989). This energy gives rise to the ionisation or excitation of the target molecules and consequently damages them.

DNA is one of the potential targets for ionising radiation both in direct and indirect ways. Direct damage can happen when the ionising radiation interacts with the DNA itself and changes its structure. On the other hand DNA in living cells is in an environment populated by numerous molecules including inorganic iron and water. These molecules can also be ionised and give rise to reactive species which are capable of interacting with DNA (Ward, 1988a).

Approximately 80% of the energy of ionising radiation deposited in living cells is consumed in the ionisation of water by the following reaction:

$$H_2O \longrightarrow H_2O^+ + e$$

Other reactive species of biological importance are formed as follows:

$$H_2O^+ + H_2O \longrightarrow {}^{\cdot}OH + H_3O^+$$
$$^{\cdot}OH + {}^{\cdot}OH \longrightarrow H_2O_2$$
$$e + + O_2 \longrightarrow O_2 -$$
$$2\,O_2- + 2H+ \longrightarrow O_2 + H_2O_2$$

Reactive oxygen species generated from the ionisation of water in living cells are the major source of indirect damage to DNA (Riley *et al.*, 1994). The most abundant DNA lesions caused by ionising radiation are oxidative base damages. Sugar damage by ionising radiation is estimated to be almost 3 times less than the damage to the bases, but by giving rise to single or double strand breaks, it is considered a biologically important lesion (Ward, 1988a). DNA strand breaks can also be generated in an indirect way as a consequence of base damage, which following the initial steps of the repair process give rise to alkaline labile abasic sites. Ionising radiations can also induce DNA strand breaks in direct way. Most of the lethal effects of ionising radiations are associated with these lesions (Hutchinson , 1985; Riley, 1994).

1.2.1.4 Other extra-cellular sources of reactive oxygen species

Apart from radiations, reactive oxygen species can be produced by other extra-cellular sources, including heat, various drugs, and chemical agents. Some tumour promoters such as 12-O-tetradecanoylphorbol-13-acetate are among these compounds (Riley, 1994; Wei_H, 1991).

1.2.2 Endogenous sources of ROS

About one billion years after the appearance of single cell organisms, oxygen was produced as a result of photosynthetic activity. Since then, oxygen began to serve as the major electron acceptor in aerobic metabolism; a breakthrough in evolution, which led to increased efficiency in ATP production. Consequently, many biological systems evolved to rely entirely upon oxygen to continue survival (Floyd, 1990a).

However, the enzymatic and chemical reduction of molecular oxygen during the aerobic metabolism, produces intermediate reactive oxygen species (ROS), which are toxic to living cells. These include hydroxyl radicals, super-oxide and singlet oxygen (Floyd *et al.*, 1986).

Leakage associated with the reduction of oxygen to water during mitochondrial respiration produces the major percentage of the intracellular reactive oxygen species. These ROS include singlet oxygen, peroxide radicals ($.O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (.OH) (Riley, P.A., 1994). Other major sources of endogenous reactive oxygen species include peroxysomal metabolism, the enzymatic synthesis of nitric oxide, the programmed release of reactive oxygen species by

macrophages in inflammatory responses, and low level background radiation (Collins, 1999).

1.2.3 Radical induced DNA lesions

No matter what their source, ROS are able to react with DNA and cause damage. These radicals differ in terms of their DNA damaging potential. Some of these radicals do not damage DNA directly, but following certain reactions they can give rise to highly reactive DNA damaging species. An example of this case is H_2O_2, which is relatively inert by itself but it can give rise to highly reactive .OH radicals by a reaction catalysed by transition metal ions, mainly Fe^{2+}. This reaction is known as the metal-catalysed Haber-Weiss reaction, or the Fenton reaction (Imlay *et al.*, 1988):

$$\text{DNA (Fe(II))} + H_2O_2 \longrightarrow \text{DNA (Fe (III))} + {}^{\cdot}OH + OH^{-}$$

There are a great variety of oxidative DNA damages caused by radical attack and as much as a hundred different DNA modifications have been associated with ROS. Many of these lesions have been characterise by treating DNA *in vitro* with ionising radiation. These include small oxidative base modifications, abasic sites, bulky adducts, and strand breaks (Moller *et al.,* 1998)(Table1.1). Bulky lesions are DNA modifications that disturb the conformation of the double helix. Examples of bulky adducts are cross links, cyclic adducts and I-compounds. Some bulky adducts result from secondary products generated in the reactions of ROS with lipids and proteins. While small oxidative base damage and strand breaks usually arise following the direct attack of ROS on DNA (Moller *et al.,*1998).

Lesion	Examples
Small oxidative base lesion	Thymine glycol, Fappy lesions,8-oxoG, 8-oxoA
Strand breaks	Single and double strand breaks
Cross links	DNA-protein, DNA intrastrand, DNA interstrand
Exocyclic adducts	εda, εdC, 1, N2-dεG, n2,3-dεG, AdG, CdG
Intracyclic adducts	8,5'-cyclopurine 2'-deoxyribonucleoside
Alkylated adducts	N7-(2-hydroxyl)-2'deoxyguanine, M1dA, M1dC
I-compounds	Unknown

Table 1.1 Oxidative DNA lesions (*Adapted from Moller and Wallin, 1998*)

As many as 35 different oxidative base modifications have been identified (McBride *et al.*,1991). These are mainly oxidation products of pyrimidines (eg. Thymine glycol), oxidation products of purines (eg. 8-oxodG, 8-oxodA) and imidazol ring fragmentation products of pyrimidines (eg. Formamidopyrimidine) (Fig.1.4). 8-oxoG and formamidopyrimidines are the most abundant lesions constituting more than 80% of the DNA oxidative base damages formed *in vitro* by hydroxyl radicals produced by the Fenton reaction of H_2O_2/Fe^{3+}. The minor products in these experiments were 8-oxodA, cytosine glycol and thymine glycol (Aruoma, *et al.*,1989). Some of the most important oxidative base damages are discussed in the following sections.

8-oxopurines

The 8-oxo-7,8-dihydropurines, (8-oxopurines), 8-oxoadenine (8-oxoA) and 8-oxoguanine (8-oxoG), are common oxidative base damages produced in DNA during normal aerobic metabolism, exposure to ionising radiations or oxidising chemical agents. Both of these lesions have been extensively studied using the radiation induced decomposition of model compounds (Cadet *et al.*,1985).

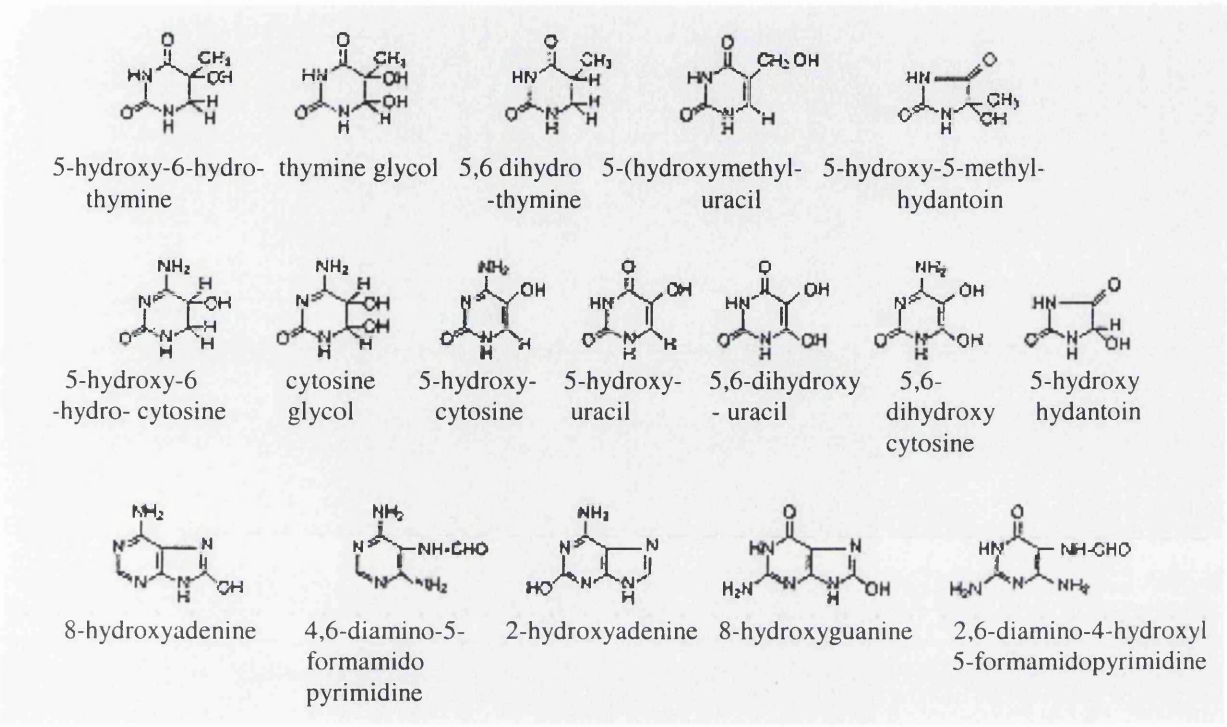
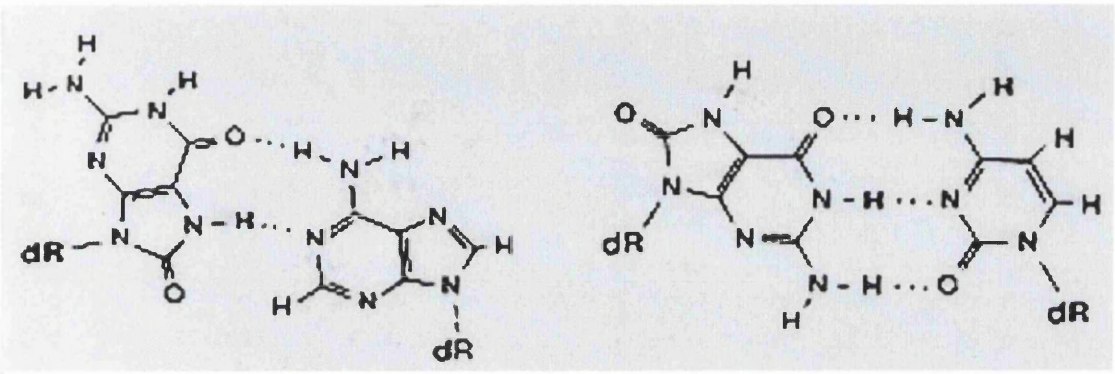


Figure 1.4 Structure of representative oxidized pyrimidine and purine bases. (*Adapted from Laval, 1996*)

Since the development of sensitive quantifying assays, provided by electrochemical detection (EC), coupled with HPLC, 8-oxoG has been used as a benchmark lesion in quantifying the oxidative base damage in many studies (Floyd *et al.*,1986; Floyd, 1990c; Kasai *et al.*,1986; Poulsen *et al.*, 1998). It has also been shown that the amount of 8-oxoG in urine is proportional to the species-specific basal metabolic rate. This finding suggest that there is a positive correlation between the metabolic rate and the accumulation and removal of this lesion from DNA (Shigenage *et al.*,1991; Poulsen *et al.*, 1998).

Biological and chemical consequences of the presence of 8-oxoguanine in DNA, have been investigated by the site specific introduction of 8-oxoG in place of C into oligonucleotides. The results indicate that 8-oxoG does not block the action of DNA polymerases (Kuchino *et al.*,1987). It was shown later that 8-oxoG is a mutagenic lesion. The first evidence of the mutagenic potential of 8-oxoG came from the observation that dAMP, as well as dCTP can be incorporated opposite 8-oxoG lesion during replication *in vitro* (Shibutani, 1991). In support of these findings, site specific introduction of 8-oxoG into a phage genome did not effect phage survival but caused a significant increase in GC to TA transversions (Wood *et al.*,1990; Moriya *et al.*,1991).

NMR and crystallographic studies of oligonucleotides containing 8-oxoG opposite cytosine or adenine demonstrated that with C opposite, 8-oxoG assumes a normal anti conformation. With A opposite, 8-oxoG assumes a syn conformation forming a stable Hoogsteen base pair with the A in the anti conformation (Fig. 1.5), This provides an explanation for the misincorporation of dAMP opposite 8-oxoG *in vivo* and *in vitro* studies (Kouchakdjian *et al.*,1991; Oda *et al.*,1991; McAuley *et al.*,1994).



8oxodG(syn).dA(anti) **8oxodG(anti).dC(anti)**

Figure 1.5 Base pairs containing 8-oxodG (*Adapted from Grollman, 1993*).

Furthermore, the geometric structure of 8-oxodG (syn) A(anti) resembles a Watson Crick base pair. Therefore the exonucleotic proof-reading activity of *E.coli* DNA polymerases fail to recognise and remove 8-oxoG:A as a mismatch (Shibutani *et al.*,1991; Grollman *et al.*,1993).

Mutagenic potential of 8-oxoG has been investigated *in vivo* by transfecting phage M13 DNA containing a single 8-oxoG lesion into the *fpg/mutY* double mutants of *E.coli* (which are unable to remove 8-oxoG). 65% of the phage progeny were mutants and there was no significant reduction in the phage transfection efficiency. Sequence analysis has confirmed that the majority of these mutations were targeted at the 8-oxoG position, more than 95% of them being GC to TA transversions. The *mutM* mutants of *Ecoli*, defective in Fpg and therefore unable to remove 8-oxoG from DNA, had a five fold increase in the frequency of spontaneous GC to TA transversion mutations (Michaels *et al.*, 1991). These mutants also show a 6 fold increase in the steady state level of 8-oxoG (Bessho *et al.*, 1992). These findings suggest that 8-oxoG is principally a mutagenic lesion with marginal or no lethal potential (Grollman *et al.*,1993).

This important mutagenic lesion, with it's high frequency of appearance in the genome contributes significantly to spontaneous mutations in genomic DNA and has been regarded as an "enemy within"(Grollman *et al.*,1993). The accumulation of 8-oxoG has been reported in cancerous tissues in some human studies and whether 8-oxoG can be used as an indicator for cancer risk assessment is a subject for further research (Helbock *et al.*, 1998, Halliwell, 1998).

Like 8-oxoG, 8-oxoA, when site specifically introduced into an oligonucleotide, is not

a block to DNA polymerases *in vitro* but it has miscoding properties. It has been shown that 8-oxoA:G mispairs occur at a very low frequency during *in vitro* DNA synthesis (Guschlbauer *et al.*,1991; Shibutani *et al.*,1993). However in a mammalian cell line NIH3TS, 8-oxoA has been shown to be as mutagenic as 8-oxoG (Kamiya *et al.*,1995).

Imidazol ring fragmentation products of purines

2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (Fapy A), have been identified in γ irradiated DNA. A scheme for the generation of these lesions is shown in Fig. 1.6. These lesions have not been as well investigated as 8-oxopurines, but it has been shown that the methylated form of Fapy-G, (Me-Fapy-G) is a potent inhibitor of DNA synthesis by *E.coli* and phage T4 DNA polymerases (Boiteux *et al.*,1983; O'Connor *et al.*,1988). Introduction of methyl Fapy G in phage M13, has resulted in a decreased transformation efficiency, indicating that me-Fapy-G blocks DNA replication *in vivo* as well as *in vitro*. Furthermore, mutation analysis experiments have revealed that the presence of me-Fapy-G (Me-fapy-G) in M13mp18 DNA gives rise to a slight increase in GC to TA and GC to CG transversion mutations following transfection into *E.coli*. These results imply that me-Fapy G and by analogy, Fapy G, are principally lethal lesions with borderline mutagenic potential (Tudek *et al.*, 1992).

Thymine glycol

5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), is one of the best studied oxidative base damages. *In vitro* studies, using purified DNA polymerases, indicate that thymine glycol can block DNA synthesis *in vitro*, suggesting that it is a lethal

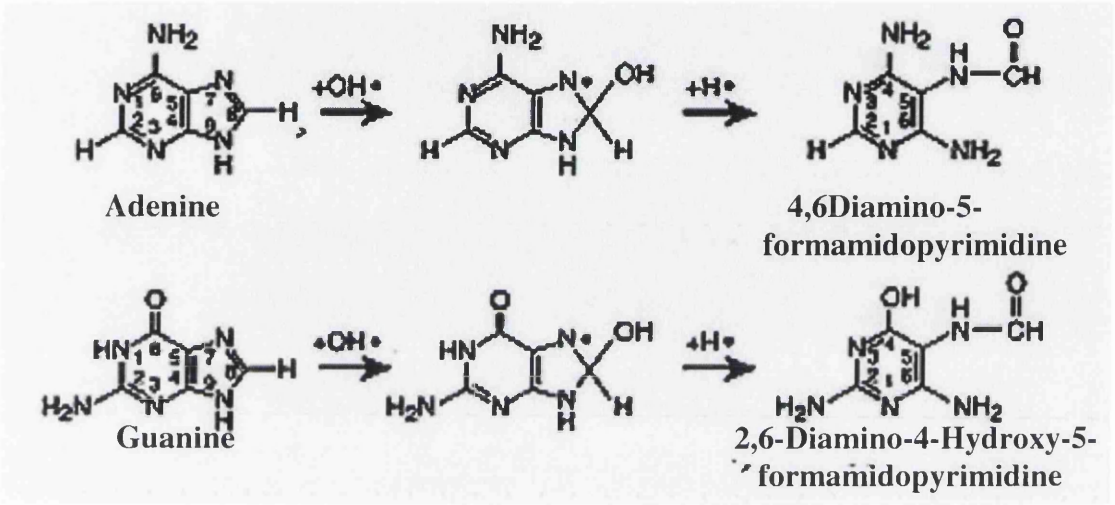


Figure 1.6 Imidazole ring opening in adenine and guanine following radical attack giving rise to the products shown. (*Adapted from Chetsanga et al.,1981*)

lesion (Demple and Harrison, 1994; Boiteux, 1993). The biological significance of this lesion has been further investigated by the transfection of OSO_4-treated phage DNA into bacterial hosts with different repair capacities. A reduction in transformation efficiency was observed in *E.coli* strains with no repair function to remove thymine glycol compared to wild type bacteria (Kow *et al.*,1990). Other studies have demonstrated that thymine glycol, when present in single stranded phage DNA, can give rise to TA to CG transitions at low frequency (Basu *et al.*,1989). These findings suggest that thymine glycol is a lethal lesion with low mutagenic potential (Girard and Boiteux, 1997).

1.2.4 Consequences of oxidative damage to DNA

Every minute as much as 10-20 litres of oxygen is pumped through our blood. Some 2-3% of this oxygen consumed by the cells is converted to highly destructive oxygen free radicals (Finkel, 1997). As discussed earlier, reactive oxygen species can cause extensive damage to DNA and other biologically significant macromolecules such as proteins and lipids. Since lipids and proteins are constantly degraded and resynthesised, the most important consequence of oxidative stress is the DNA modifications that can give rise to mutations and other types of genomic instability. Therefore, all living organisms have evolved defensive mechanisms to protect their genome against ROS attacks. In eukaryotes, the organelles are compartmentalised; therefore the sensitive target molecules such as DNA are shielded from the main sources of ROS such as mitochondria, chloroplasts and perixosomes, where active oxygen metabolism takes place (Riley, 1994). DNA surrounded by histones and organised into higher order chromatin structure is further protected from ROS

(Ljungman and Hanawalt, 1992). Antioxidant enzymes such as catalase and superoxide dismutase (Sun *et al.*, 1998), and radical scavengers like vitamins C and E, glutathione α-tocopherol, albumin, bilirubin and urate (Sies *et al.*, 1992; Collins, 1999) eliminate reactive oxygen species in living cells. Sequestration of transition metals, which involves formation of complexes between the metal in its ionic state (eg. Fe^{2+} and Ca^{2+}) and reactive oxygen species are other mechanisms to prevent the chemical effect of ROS in the cells (Friedberg, 1995). Furthermore, the presence of reactive oxygen species can trigger cellular responses such as inhibition of cell cycle progression and the initiation of apoptosis (or programmed cell death) (Goel and Khanduja,1998). Oxidative stress responses of *S. cerevisiae* are reviewed in Jamiesson, (1998).

Under normal physiological conditions, the level of cellular ROS is regulated by antioxidants produced constantly in cells. However, interruption of cellular redox homeostasis and an elevated flux of oxygen radicals into cells leads to oxidative stress, which can have serious consequences. Damage to DNA, proteins and lipids by reactive oxygen has been suggested to be one of the major contributors to degenerative diseases such as cancer, ageing, neurological degeneration, and cardiovascular diseases (Ames *et al.*,1993; Wei-YH 1998, Sun and Chen, 1998; Chakraborti, 1998).

Oxidative DNA damage by ROS can cause mutations and by activating tumour promoter genes or inactivating tumour suppresser genes can cause cancer. The ras family, proto-oncogenes, and the p53 tumour suppresser genes are the most well known cancer related genes. Frequent occurrence of G to T transversion mutations, caused by misincorporation of 8-oxoG lesions produced by ROS in DNA, has been reported in the middle position of a hot spot codon 12 in Ki-ras and H-ras in non

melanoma skin tumours (DayaGrosjean *et al.*,1993). Some of the G to A transversions observed in the p53 gene in nonmelanoma skin tumours, could have been caused in the same way. G to T transversions in many p53 codons have also been observed in smoking related lung carcinomas (Takahashi *et al.*,1989) and hepatocarcinomas (Bressac *et al.*,1991). C to T and G to A transitions produced by deamination of 5-methyl cytosine by ROS attack comprises nearly 95% of the mutations at the p53 hot spot codon 248 (CGG) in colerectal carcinoma (Nguyen *et al.*,1992).

Accumulation of oxidative damage to DNA, proteins and other macromolecules with age has been suggested as an important factor leading to ageing . In fact, it has been argued that the life span of organisms depends on their species-specific metabolic rate, which is correlated to the amount of accumulated oxidative damage. It has been estimated that everyday, as many as ten thousand oxidation reactions that cause DNA damage occur in each human cell. Rats have a higher rate of metabolism and a much shorter life span than humans. They receive almost 10 times more oxidative 'hits' and it is estimated that a 2 years old rat probably has accumulated about 2 million DNA lesions in every cell (Ames *et al.*,1989).

As well as the natural accumulation of oxidative damages with time, an age related decline in antioxidant defence mechanisms can give rise to increased levels of oxygen free radicals, and as a result, increased levels of oxidative damage to molecules such as DNA. Mitochondrial DNA is a particularly sensitive target for reactive oxygen species as it is located at the site of active oxidative metabolism. Having no NER (Clayton *et al.*, 1974; Bohr and Dianov, 1999), the presence of single stranded DNA in its characteristic constantly replication state, and lacking histones, all make

mitochondrial DNA more vulnerable to ROS attack than nuclear DNA (Ames, 1989; Wei_YH, 1998). It has been reported that mitochondrial DNA from rat liver accumulates 10 times more oxidative DNA damage than does nuclear DNA from the same tissue (Richter *et al.*, 1988; Richter *et al.*,1992). Oxidative damage to mitochondrial DNA results in mutations in the genes encoding the enzymes involved in cell respiration. These mutations accumulate with age and ultimately give rise to mitochondrial failure, energy depletion and functional degeneration of the old cells (Finkel, 1997; Lenaz, 1998). Brain cells in particular suffer a great deal because of their tremendous energy requirement. Therefore, oxidative stress due to the excessive production of free radicals in the cells, or a deficiency in antioxidant defence mechanisms have been suggested to be central to ageing and to age-related neurodegenerative diseases such as Parkinson's and Alzheimer's (Sun and Chen, 1998). Oxidative damage to DNA has also been implicated in pathophysiology of diseases such as heart diseases, diabetes and cataracts. (Chakraborti *et al.*, 1998; Dandona, 1996). Whether oxidative stress is the primary etiological factor or is produced as a consequence of the tissue damage in these cases, is yet to be investigated (GonzalezFraguela, 1999).

1.3 DNA repair

In order to maintain the integrity of the genetic information, all living organisms have various types of repair mechanisms. These repair systems or more generally, cellular responses to DNA damages, can be grouped into three broad categories shown below. Through these mechanisms, cells may completely restore the normal DNA sequence by reversing or repairing the damage, or they may minimise the effect of the damage

by tolerance mechanisms. These pathways are summarised as follows:

- **Direct reversal of damage**: Enzymatic photoreactivation and the repair of alkylation damage by alkyltransferases.

- **Excision of DNA damage** : Base excision repair, Nucleotide excision repair, Mismatch repair

- **Tolerance of DNA damage**: Replicative bypass of template damage with gap formation and recombination. Translesion DNA synthesis.

1.3.1 Direct reversal of the damage

The best studied mechanism of reversal of damage is the monomeraization of cylobutane pyrimidine dimers by the Phr enzyme in *E.coli* (Sancar 1988). The photoreactivation processes starts by binding of the photolyase enzyme to CPDs in the dark. Following excitation with a blue light photon, the enzyme then splits the cyclobutane ring restoring the two pyrimidines into their original form. Subsequently the enzyme is released from the DNA (Hearst, 1995) (Fig. 1.7). The *S.cerevisiae* photolyase is encoded by the *PHR1* gene. It has 36.2% amino acid homology with the *E.coli* enzyme and is capable of complementing *phr-* mutants. There is evidence in both organisms that photolyase enzyme can promote the removal of CPDs by NER in the absence of photoreactivating light (Sancar and Smith, 1989).

Another example of reversal of damage is the repair of alkylated bases such as O_6-

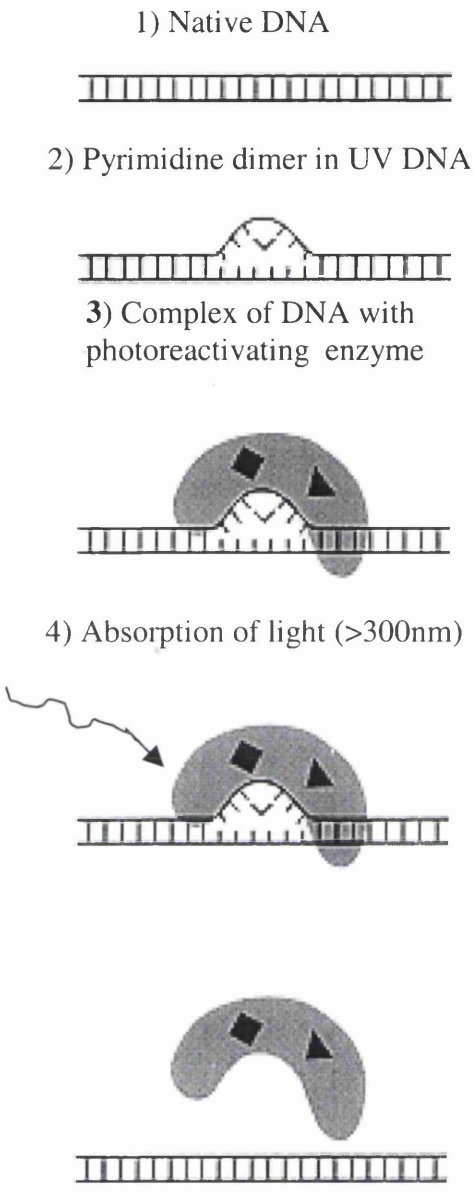


Figure 1.7 Enzyme catalyzed monomerization of pyrimidine dimers by photolyase (photoreactivating enzyme). The square and triangle symbols represent the two chromophores essential for catalytic activity in all DNA photolyases.

(*Adapted from Friedberg, 1985*)

alkylguanine and O_4-alkylthymine by alkyltransferases. The repair mechanism involves the direct transfer of an alkyl group from the DNA bases to a cystine residue within the alkyltransferases. Alkyltransferases are apparently present in all living organisms (Samson, 1992; Guzder *et al.*, 1991).

Although direct reversal of damage could be an easy way to correct the DNA damages, in most cases it is not thermodynamically possible.

1.3.2 Excision repair mechanisms

1.3.2.1 Base excision repair (BER)

Base excision repair is the most commonly employed pathway to remove incorrect bases in DNA and it consists of the following steps (Friedberg *et al.*, 1995; Krokan *et al.,* 1997) (Fig.1.8a , Fig.1.8b).

1. Cleavage of the glycosylic bond between the damaged base and the sugar phosphate backbone by a DNA glycosylase.

2. Removal of AP sugar by an AP endonucleases/AP lyase.

3. Removal of the 5' terminal deoxyribose phosphate residue by a dRpase.

4. Replacement of the missing nucleotide by a DNA polymerase and ligation of the nick by a DNA ligase.

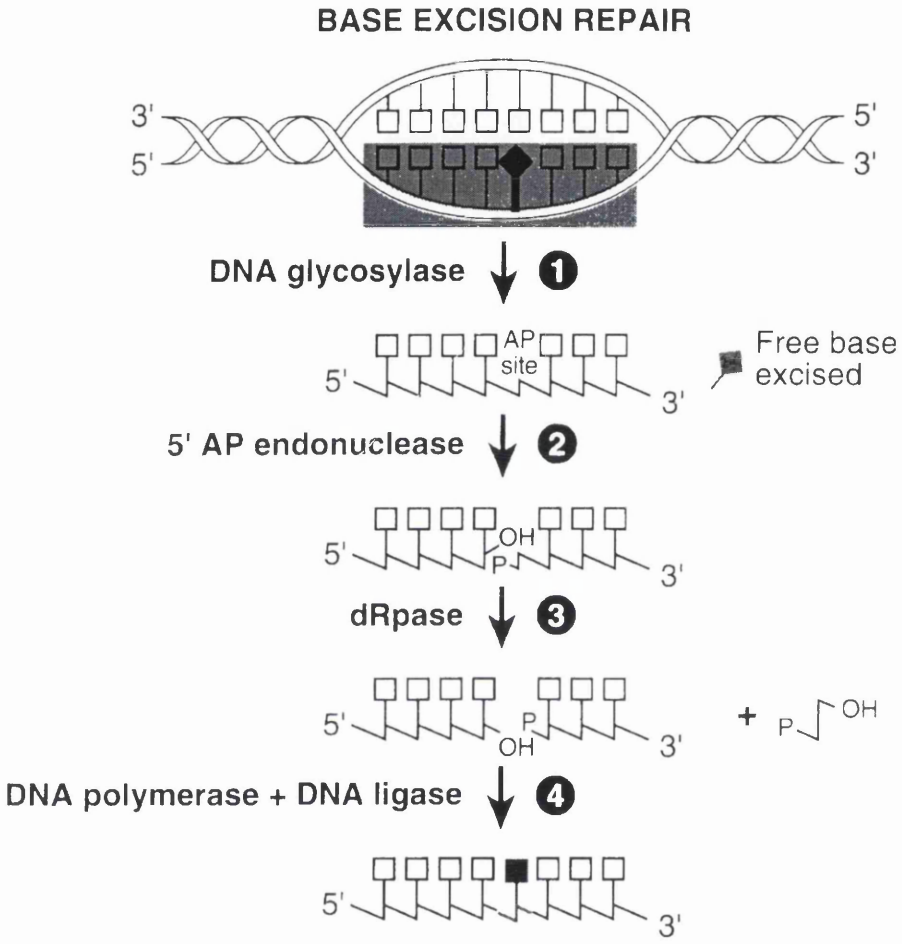


Figure 1. 8a Schematic representation of base excision repair basic processes. (*reproduced from Friedberg et al., 1995*)

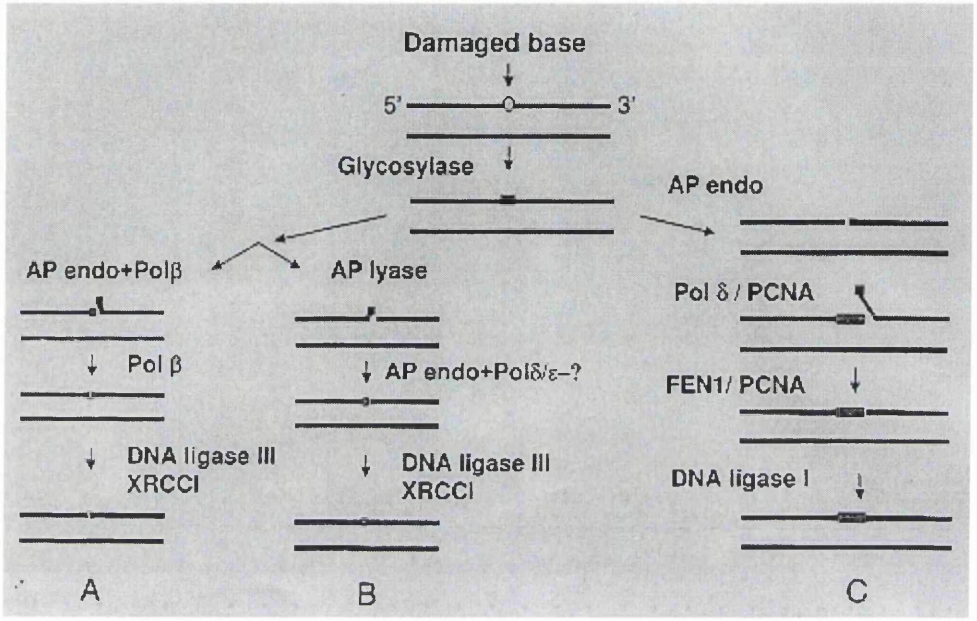


Figure 1. 8b Branched base excision repair pathway in human. **A.** Single –nucleotide patch repair pathway initiated by glycosylases without AP lyase activity. **B.** Single nucleotide patch repair pathway initiated by glycosylase with AP lyase activity. **C.** Long patch repair pathway replaces two to six nucleotides instead of only one as it happens in short patch repair (*Adapted from Bohr and Dianov, 1999*).

1.3.2.1.1 Excision of the damaged base-DNA glycosylases

Hydrolysis of the N-glycosylic bond which links the damaged base to the deoxyribose phosphate backbone is the first step in BER. This reaction is catalysed by a particular class of enzymes called DNA glycosylases (Fig. 1.9)(Lindahl, 1979). These are small proteins, which are usually less than 30 kDa in size. The majority of DNA glycosylases are highly specific for a particular type of base damage. Some of the known DNA glycosylases are listed in the table 1.2.

DNA Glycosylase	**Substrate**
Ura DNA glycosylases	DNA containing uracil
Hmu DNA glycosylase	DNA containing hydroxymethyl uracil
5-mc-DNA glycosylase	DNA containing 5- methylcytosine
Thymine mismatch DNA glycosylase	DNA containing G.T mispair
MutY DNA glycosylase	DNA containing G.A mispairs
3-mA-DNA glycosylase I	DNA containing3-methyladenine
3-mA-DNA glycosylaseII	DNA containing3-methyladenine,7-methylguanine,or3 methylguanine
Fapy-DNA glycosylase	DNA containing Formamidopyrimidine or8-hydroxyguanine

Table 1.2 Examples of DNA glycosylases (Friedberg *et al.*, 1995)

As a result of the hydrolysis reaction by DNA glycosylases, the damaged bases are excised as free bases leaving apurinic or apyrimidinic (AP) sites. AP sites can also be

generated by spontaneous hydrolysis of the N-glycosylic bonds. No matter how they arise, these sites of base loss are considered as another form of DNA damage and they have to be removed by further repair processes, which will be discussed shortly. DNA glycosylases have been classified into two groups. These include pure DNA glycosylases with the sole function of removing the damaged base, and the DNA glycosylases with associated DNA nicking activity at AP sites. These glycosylases have been described as AP lyases (Fig 1.8b).

1.3.2.1.2 Removal of AP sites

5' AP endonucleases

The removal of AP sites is an important biochemical event during BER and it requires the action of the second class of BER enzymes called AP endonucleases (Fig. 1.9) which specifically recognise AP sites in double stranded DNA. These enzymes then catalyse the hydrolysis of the phosphodiester bonds immediately 5' or 3' to each AP site producing an incision or nick in duplex DNA. Most AP endonucleases have affinity for the 5' phosphodiester bond and are called 5' AP endonucleases (Lindahl, 1979; Krokan *et al.*, 1997).

Removal of the 5' terminal deoxyribose phosphate residue - dRpase activity by exonucleases

Hydrolysis of the phosphodiester bond immediately 5' to an AP site by 5' endonucleases generates a 5' terminal deoxyribose phosphate residue (Fig. 1.9). A third class of BER enzymes, called exonucleases, are responsible for removing such residues. The exonuclease enzymes have the ability to degrade DNA with free ends in

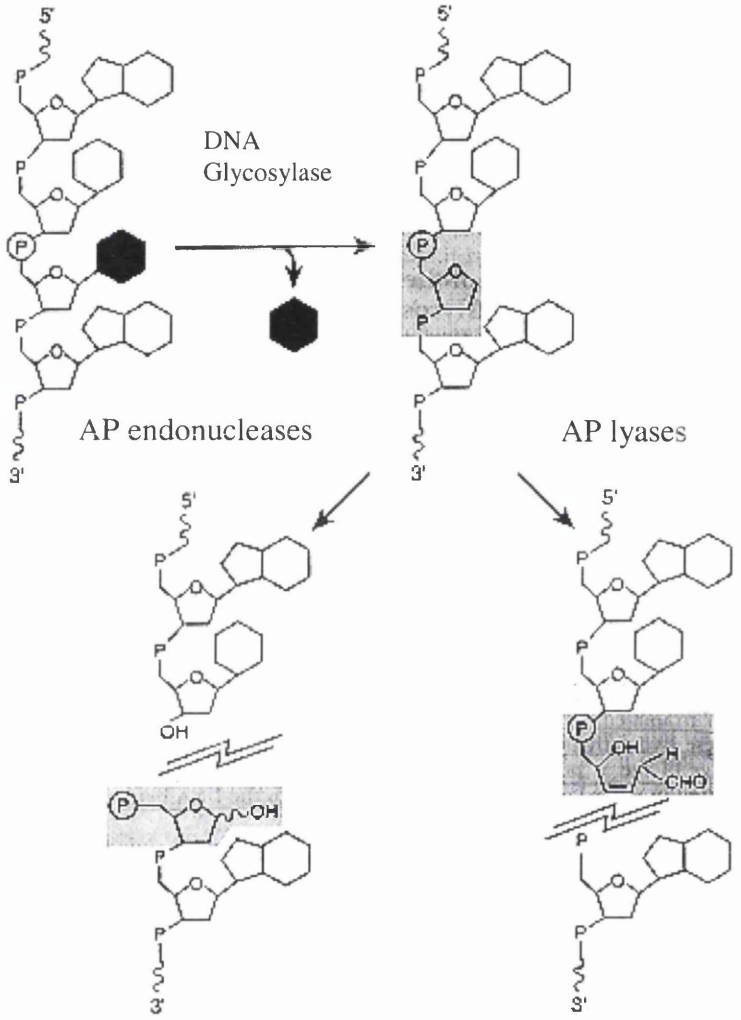


Figure 1.9 Phosphodiester bond cleavage sites at an AP site by AP endonucleases and AP lyases. AP endonucleases cleave hydrolytically 5' to AP sites yielding 5' deoxyrobose-5-phosphate (gray box) and 3'-OH nucleotide residues. AP lyases cleave by β elimination yielding 5'-terminal deoxynucleoside-5'-phosphate residues and 3'-terminal α,β-unsaturated aldehydes (gray). (*Adapted from Demple and Harrison, 1994*).

different events such as repair, recombination, and replication and, unlike DNA glycosylases or AP endonucleases, they are not limited to repair-only functions. The 5' terminal deoxyribose phosphate residues discussed above, are removed by a family of exonucleases called deoxyribophosphodiesterases (dRpases). These enzymes leave a single nucleotide gap in the double stranded DNA (Friedberg *et al.,*1995; Krokan *et al.*, 1997).

DNA glycosylases with associated AP lyase activity

It has been shown that the incubation of some DNA glycosylases with substrate DNA *in vitro* results in the breakage of the phosphodiesterase bond. Further investigations have shown that the DNA incision by all the DNA glycosylases capable of incising the double stranded DNA occurs by a β elimination immediately 3' to the AP site (Bailly *et al.,*1987; Mazumder *et al.,*1991; Krokan *et al.*, 1997).

In order to avoid the controversy in definition and distinction of true AP endonucleases from DNA glycosylases with AP endonuclease activity, it has been suggested that these DNA glycosylases that can also incise DNA by a β elimination reaction should be called AP lyases rather than AP endonucleases (Friedberg *et al.,*1995).

β elimination by AP lyases generates a strand break which requires further processing to provide a free 3' OH for the subsequent action of DNA polymerases(Doetsch and Cunningham, 1990).

1.3.2.2 Base excision repair enzymes of *E.coli*

DNA repair pathways for oxidative base damage have been best studied in *E.coli*. In

summary, the oxidative DNA damage in *E.coli* is principally repaired by the base excision repair pathway (BER) employing four DNA glycosylases; endonuclease III and endonuclease VIII for the repair of oxidised pyrimidines and Fpg and Mut Y for the repair of oxidised purines (Demple and Harrison, 1994; Boiteux *et al.*,1993; Grollman and Moriya, 1993; Girard and Boiteux, 1997; Cunningham, 1997; Boiteux and Radicella, 1999).

Endonuclease III of *E.coli* (NTH protein)

Endonuclease III was first identified in the late 1970's as an enzyme with the ability to degrade UV – irradiated DNA (Radman, 1976). Further investigations showed that the enzyme is a DNA glycosylase with associated AP lyase activity. Endonuclease III, which was independently discovered in different laboratories, is designated in literature by many different names. These include thymine glycol DNA glycosylase, urea DNA glycosylase, redoxy endonuclease, X-ray endonuclease and UV endonuclease (Doetsh and Cunningham, 1990; Friedberg *et al.*,1985).

It has been shown that this enzyme has an unusually broad substrate specificity, a characteristic untypical of most DNA glycosylases. Endonuclease III can recognise ring saturation, ring fragmentation or ring contraction products of pyrimidines exposed to oxidising agents These include thymine glycol, 5,6 dihydrothymine, uracil glycol and 5,6-dihydrouracil (Boorstein *et al.*,1989; Breimer and Lindahl, 1984). 5-hydroxy-2'deoxyuridine and 5-hydroxy-2'deoxycytidine residues can also be recognised by this enzyme (Hatahet *et al.*,1994).

Endonuclease III is encoded by the nth^+ gene of *E.coli* (Asahara *et al.*,1989;

Cunningham and Weiss, 1985). The cloned gene has been overexpressed and the protein has been purified to homogeneity. It has been shown that this protein is an iron sulphur protein with a $(4Fe\text{-}4S)2^{+}$ cluster (Cunningham *et al.*, 1989).

The *nth* mutants do not show abnormal sensitivity to killing by hydrogen peroxide or γ radiation (Cunningham and Weiss, 1985). However, they represent high mutation rates possibly due to the inability of the mutants to excise base damages, which can give rise to mispairings during DNA replication (Weiss *et al.*, 1988; Laval, 1996; Boiteux, 1993; Mol *et al.*, 1995c).

Endonuclease VIII (NEI protein)

Following the availability of the *nth* mutants unable to remove thymine glycol lesions, a new enzyme, later called endonuclease VIII or Nei, was identified with the function to remove thymine glycol as well as oxidised cytosine. The enzyme has been purified and shows many similarities to Nth protein. Thymine glycol, dihydrothymine, and some urea residues are substrates for this enzyme. Nei protein has also the ability to incise DNA at abasic site by β lyase activity. It is speculated that this enzyme has a back up role for Nth protein (Melamede *et al.*, 1994).

The Fpg protein (Formamidopyrimidine DNA glycosylase)

The Fpg protein was first characterised in *E.coli* in association with its DNA glycosylase activity releasing the ring open form of 7-methylguanine from methylated, alkali-treated DNA *in vitro* (Chetsanga *et al.*, 1979). Subsequently, it was shown that Fpg can also remove imidazol ring ruptured residues of adenine (Breimer, 1984).

The *fpg* (*mutM*) gene of *E.coli* was cloned and the enzymatic and physical characteristics of the protein have been studied in great detail (Boiteux *et al.*,1987; Boiteux *et al.*,1992; Boiteux *et al.*, 1990). Fpg protein is a globular monomer of 30.2 KDa and contains one zinc atom per molecule. Studying the amino acid sequence of the Fpg protein indicates a Zinc finger consensus sequence (Boiteux *et al.*,1990). The zinc finger is essential for functional activities of the enzyme, since mutants of the zinc finger domain have neither a DNA glycosylase nor a β lyase activity (O'connor *et al.*,1993).

The DNA glycosylase activity of Fpg protein recognises a broad range of substrates. Apart from the Fapy residues (Boiteux *et al.*,1987; Bioteux *et al.*,1990; Laval *et al.*,1990), it excises 8-oxoG, (Boiteux *et al.*,1992; Tchou *et al.*, 1991), 8-oxo-A and the imidazol ring open form of Guanine and Adenine (Boiteux *et al.*,1992). Using oligonucleotides with a single modified base at a specific position, the removal of 8-oxoG by the Fpg enzyme, has been examined. These studies reveal that the enzyme has the highest rate of excision for 8-oxoG when paired with C or T, and 5 and 200 fold slower rate of excision, when 8-oxo G is paired with G and A respectively (Castaing *et al.*,1993; Tchou eta l., 1991; Boiteux, 1993).

Besides it's glycosylase activity, Fpg enzyme has two other enzymatic activities. First, it cleaves phosphodiester bonds both 3' and 5' to the AP sites leaving a one nucleotide gap (O'connor *et al.*,1989); second, Fpg enzyme is endowed with a dRpase activity which removes the deoxyribose 5 phosphate residues generated following the sequential action of DNA glycosylases and AP endonucleases (Graves *et al.*,1992) (Reviewed in Laval *et al.*, 1996; Girard and Boiteux, 1997).

The Mut Y protein of *E.coli*

Mut Y protein, encoded by the *mutY* gene of *E.coli*, is a DNA glycosylase, which excises adenine residues when mismatched with G or 8-oxoG (Tsai, *et al.*,1992). Investigation of its amino acid sequence reveals an iron sulphur centre like the NTH protein (Michaels *et al.*,1990). A human homologue of MutY has been identified which can be recognised by anti Mut Y antibodies of *E.coli* (McGoldrick *et al.*,1995; Laval, 1996; Girard and Boiteux, 1997)

The Mut T protein (8-oxo-dGTP triphosphatase)

8-oxo-dGTP which can be inserted opposite dA or dC with equal frequency during replication, arises as a result of oxidation of dGTP. This residue is degraded very efficiently by Mut T protein to the original dGTP monophosphate, ensuring a very low frequency of this mutagenic lesion in the nucleotide pool. The *mutT* mutants of *E.coli* show a high frequency of A:T to C:G transversion mutations (Maki *et al.*,1992).

Fig. 1.10, Shows the removal of 8-oxoG by *E.coli* BER enzymes.

1.3.2.3 Base excision repair enzymes in *Saccharomyces cerevisiae*

The *OGG1* gene of *S.cerevisiae*

It has been demonstrated that the transformation of the highly spontaneous mutator *fpg/mutY* double mutant strain of *E.coli*, by a yeast DNA library results in the formation of colonies with reduced spontaneous mutation frequencies. These

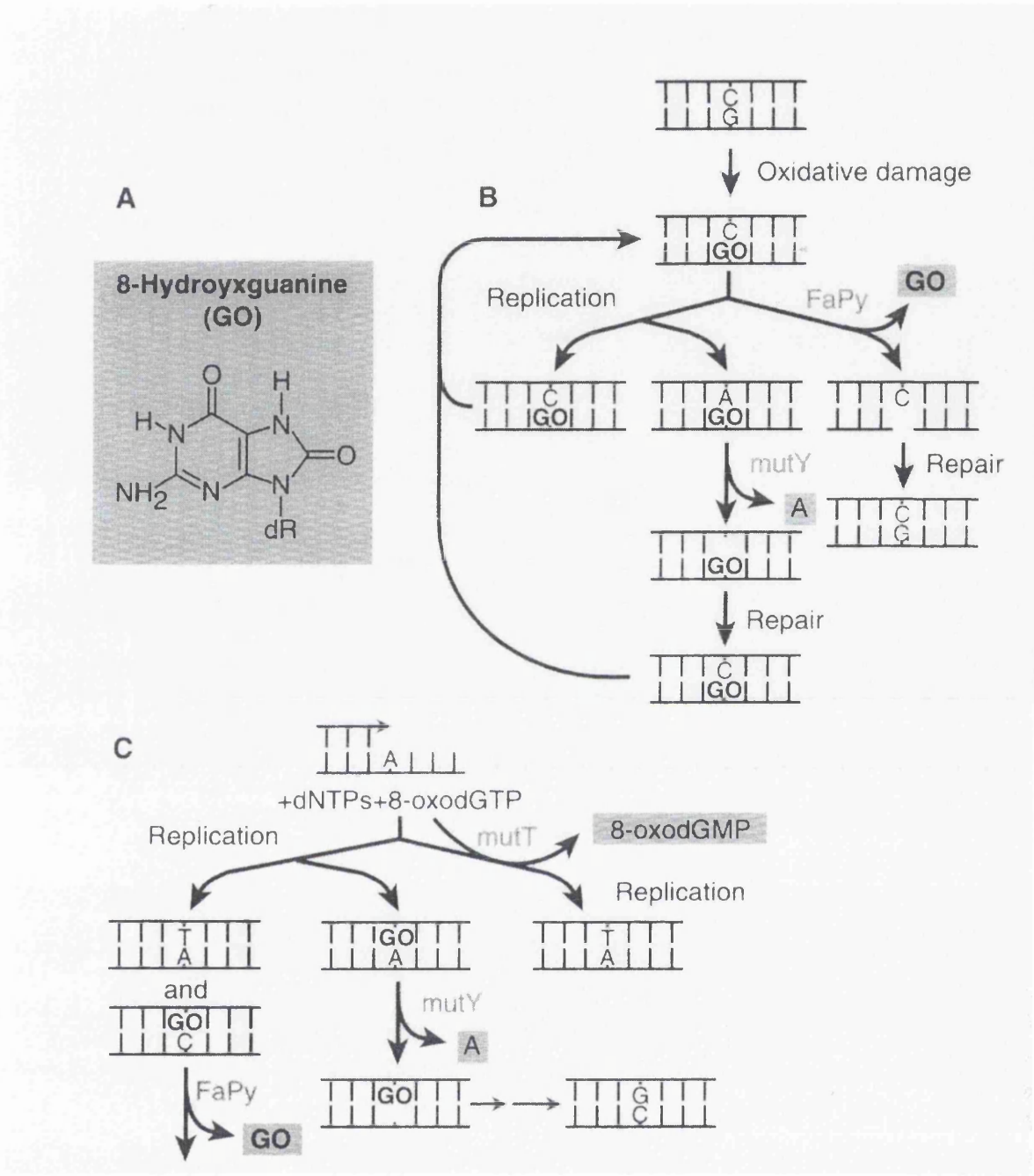


Figure 1.10 Schematic representation of the enzymatic removal of 8-oxoG (represented as GO) by BER enzymes of *E.coli*. (A): GO in its predominant tautomeric form. (B right): The excision of 8-oxoG by Fapy DNA glycosylase (Mut M) protein and restoring the normal G.C base pair by subsequent steps of BER. (B left): When the 8-oxoG is not removed prior to DNA replication, DNA synthesis can retain the GO.C base pair which can be repaired by a subsequent opportunity as before. (A center): Alternatively DNA replication may give rise to a GO.A mispair which is a substrate for MutY DNA glycosylase. This can replace the A with C, giving rise to GO.C which has again another opportunity to be repaired by Fapy DNA glycosylase. (C top right) : Oxidative damage can produce 8-oxo-dGTP in the triphosphate pool. This is a substrate for MutT. (C left) If 8-oxo-dGTP is not removed prior to replication, replication would be most likely correct , as T preferentially pairs with A. However, if replication give rise to the formation of GO.C, this can be repaired by Fapy DNA glycosylase as before. (C center): If replication give rise to GO.A mispair, repair by Mut Y DNA glycosylse leads to an AT to GC transition mutation. (*Adapted from Michaels and Miller, 1992*).

transformed colonies were harboring pYSB10, a 11kbp recombinant plasmid. Subsequently, it was shown that cell free extract from these *fpg/mutY* strains transformed with pYSB10, has an enzymatic activity capable of cleaving a 34 mer oligonucleotide with a single 8-oxoG:C mismatch. Following these findings, the sequence of the 1.7 kbp yeast DNA fragment, carried by pYSB10, responsible for the suppression of the spontaneous mutagenesis and the 8-oxoG/C cleaving activity was determined. The newly recognised open reading frame was designated *OGG1* and mapped to chromosome XIII (Van Der Kemp *et al.*, 1996).

OGG1 encodes a protein of 376 amino acids with a molecular mass of 43kDa. In order to purify the Ogg1 protein, the plasmid pYSB110 made by insertion of the *OGG1* gene into pUC19, was cloned into *fpg E.coli*. The Ogg1 protein was purified to apparent homogeneity. The amino acid sequence of the protein contains a bipartite nuclear localisation signal at the carboxy terminal (Van Der Kemp *et al.*,1996; Girard and Boiteux, 1997).

The Ogg1 protein is endowed with a DNA glycosylase activity to excise 8-oxoG and 2-6 diamino-4hydroxy-5-N methyl Formamidopyrimidine (FAPY) lesions in DNA (Van Der Kemp *et al.*, 1996).

The substrate specificity and the catalytic mechanism of Ogg1 have been studied in detail. These studies indicate that Ogg1 has a remarkable preference for the 8-oxoG or the AP sites opposite a cytosine. The DNA glycosylase and AP lyase efficiency of the Ogg1 protein is significantly reduced when 8-oxoG is placed opposite T, G or A in the rank order (Girard *et al.*,1997; Van Der Kemp *et al.*,1996; Girard and Boiteux, 1998).

Recently it has been shown that besides its DNA glycosylase/Ap lyase activity, the yeast Ogg1 also has a dRpase activity to remove the deoxyribose 5' phosphate residue via a β elimination reaction. It has been suggested that in the presence of $MgCl_2$, the Ogg1 protein is able to processes 8-oxoguanine lesions so efficiently that only a one nucleotide gap is left ready for the action of DNA polymerases (Sandigursky *et al.*,1997).

The biological significance of the Ogg1 enzyme has been demonstrated by construction of *ogg1* mutants in which the coding sequence of the *OGG1* gene has been partially deleted by insertion of other DNA sequences carrying marker genes. The disruption of the *OGG1* gene does not have any effect on the viability of the mutants. The cell free extracts provided from the *ogg1* mutants do not have any detectable DNA cleaving activity on a 34mer DNA fragment containing a single 8-oxoG:C mismatch. These studies also showed that *ogg1* mutants are not hypersensitive to DNA damaging agents such as UV light, hydrogen peroxide or methyl methansulfonate, but that they display a mutator phenotype. It has been reported that the frequencies of the spontaneous mutation to canavanine resistance and reversion to Lys+ have increased seven and ten fold respectively in *ogg1* mutant strains. Furthermore, by using a specific tester system, it has been demonstrated that the *ogg1* mutants exhibit a 50 fold higher spontaneous GC to TA transversions compared to wild type (Thomas *et al.*,1997).

These findings suggest that yeast Ogg1 is the functional homologue of the bacterial Fpg protein. However, the amino acid sequence of Ogg1 and Fpg have little

similarity. A highly conserved sequence at the amino terminal of the Fpg protein and the zinc finger motif at it's carboxy terminal are absent in the Ogg1. These findings suggest that it is unlikely that the FPG gene in *E.coli* and the *OGG1* gene in *S.cerevisiae* share a common ancestral gene (Girard *et al.*,1997; Girard and Boiteux; 1997). A human homologue of the yeast *OGG1* gene has recently been cloned and characterised. The predicted 345 amino acid protein has 38% sequence identity with the yeast protein. The enzymatic activities of this protein designated hOgg1 are similar to that of the yeast Ogg1 protein and the coding sequence of hOgg1, when expressed in *ogg1* mutants of *S.cerevisiae*, is able to complement the spontaneous mutator phenotype (Radicella *et al.*,1997)

The *NTG1* gene of *Saccharomyces cerevisiae* (the yeast Fapy DNA glycosylase gene)

The *NTG1* gene was identified as a result of searching databases to find a helix bend helix motif characteristic of many DNA binding proteins. The *NTG1* gene was mapped to chromosome I of *S.cerevisiae*. The gene has been cloned and the Ntg1 protein has been partially purified. The Ntg1 is a protein of 399 amino acids with a relative molecular mass of 45kDa (Eide *et al.*, 1996).

The Ntg1 protein has DNA glycosylase and AP lyase activity, which can excise methyl Fapy guanine (Me-Fapy –G) and cleave DNA containing thymine glycols. It has been reported that the purified Ntg1 protein excises another oxidatively damaged pyrimidine, 5,6-dihydrouracil (DUH) from DNA *in vitro*. The Ntg1 protein does not have any cleaving activity on double stranded DNA containing 8-oxoG:C mismatches

(Eide *et al.*, 1996; Augeti *et al.*,1997).

The Ntg1 protein exhibit significant sequence homology with endonuclease III of *E.coli* but unlike endonuclease III, it doesn't contain an iron sulphur (4Fe-4S) DNA binding site and despite the sequence homology, the substrate specificity of two proteins differs. Both proteins can release oxidised pyrimidines, but the Ntg1 can also release methyl-Fapy-G (Me-FAPY-G) like Fpg, while endonuclease III has no activity on this substrate (Eide *et al.*, 1996).

The biological significance of the Ntg1 has been studied by disruption of the *NTG1* gene. The *ntg1* mutants are viable but they do show a moderate sensitivity to oxidising agents like H_2O_2 and menadione (Eide *et al.*,1996).

A Fapy- DNA glycosylase has been described by De Oliveira *et al.*, (1994), which excises Me-Fapy-G. The partially purified protein has a relative molecular mass of 40 kDa and possesses AP lyase activity. It can also cleave a DNA fragment containing 8-oxoG placed opposite a Guanine at a very low rate. Considering the similar substrate specificities observed from the purified Ntg1 protein, it has been suggested that the Fapy DNA glycosylase reported by De Oliveira *et al.*,is the product of the *NTG1* gene (De Oliveira *et al.*,1994; Girard and Boiteux, 1997; Boiteux and Radicella., 1999).

The *NTG2* gene

Exploring the sequence databases using the Ntg1 and Ogg1 amino acid sequences has led to the identification of the *NTG2* gene locus located on chromosome XV. The *NTG2* gene has been cloned and the Ntg2 protein has been purified to apparent homogeneity. Ntg2 protein exhibits a 41% homology to Ntg1 and limited identity

with Ogg1. The Ntg2 protein contains 380 amino acids with a relative molecular mass of 43.7 kDa and contains a 4Fe-4S motif at it's carboxy-terminal end.

Ntg2 is a DNA glycosylase/Ap lyase enzyme but it's efficiency to remove oxidised pyrimidines has not yet been revealed. *NTG2* disruption mutants of *S.cerevisiae* have been made and are viable. The biological function of the Ntg2 protein is yet to be determined (Girard and Bioteux *et al.*, 1997).

The *OGG2* gene

The recently identified yeast Ogg2 protein has been suggested to be the second yeast 8-oxoG DNA glycosylase. Its substrate specificity differs from Ogg1, and it might be involved in preventing the incorporation of 8-oxo dGTP into DNA during replication. It has also been suggested that Ntg1 is coded by the same gene as Ogg2 (Bruner *et al.*, 1998).

A common ancestor gene for the bacterial and yeast BER genes

Ogg1, Ntg1, and Ntg2 from *S.cerevisiae* are all endowed with DNA glycosylase/Ap lyase activity. They all excise Me-Fapy G and they all use β elimination to incise DNA at AP sites. These functional similarities suggest the presence of a common ancestral origin for these BER genes and the endonuclease III of *E.coli*. The coding sequences for at least 17 proteins with bacterial, eukaryotic and archibacterial origin including the Ogg1, Ntg1 and Ntg2 from *S.cerevisiae* have been compared with the coding sequence of the endonuclease III of *E.coli*. These studies have led to the identification of a consensus sequence spanning two conserved residues separated by

11 amino acids. Two amino acids directly involved in the catalytic mechanism of endonuclease III of *E.coli* K120 and D138 are conserved in all of these sequences.

It has been suggested that Ogg1, Ntg1 and Ntg2 belong to an extended family including eubacterial, archeabacterial and eukaryotic proteins which share a common ancestor with the Endonuclease III of *E.coli* (Thayer *et al.*, 1995; Girard and Boiteux , 1997).

A model for the catalytic mechanism of yeast DNA glycosylase /AP lyases

It has been suggested that the catalytic action of the *OGG1* gene in *S.cerevisiae,* involves the formation of a covalent enzyme–DNA intermediate. Comparison between the Ogg1 and Endonuclease III sequences suggests that the lysine 241 of Ogg1, corresponding to the lysine 120 of Endonuclease III, is essential in the formation of this covalent intermediate since it's substitution by glutamine via site specific mutagenesis inactivates the catalytic activity of Ogg1. Nevertheless, the Ogg1 enzyme in these mutants is still able to bind to 8-oxoG; suggesting that lysine 241 has a functional rather than a structural role in the catalytic activities of the Ogg1 enzyme (Girard *et al.*, 1997; Girard and Boiteux, 1997; Boiteux and Radicella, 1999).

The mechanism of base excision repair enzymes remains to be investigated in more depth, but the following processes have been proposed for the catalytic mechanism of the DNA glycosylase/AP lyases of *S.cerevisiae.* (Fig.1.11 shows steps 4-8 of the following scheme)

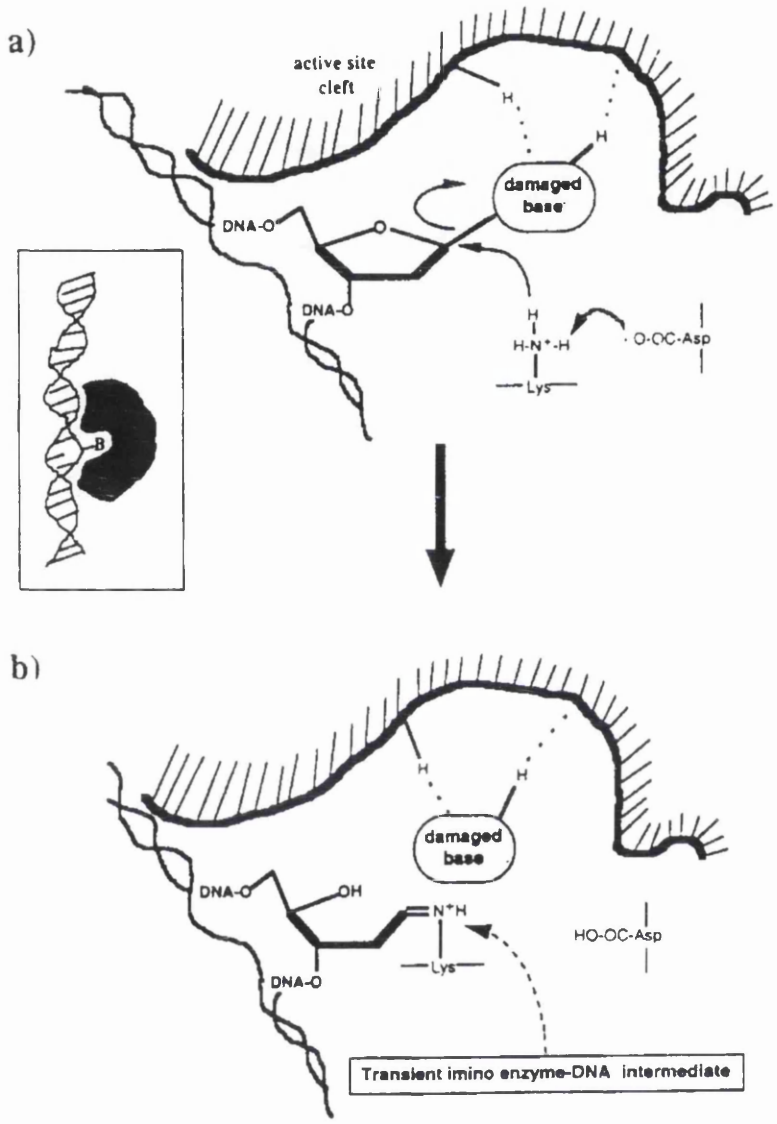


Figure 1. 11 Catalytic mechanism for the excision of oxidised DNA bases by the Ogg1 protein of *S.cerevisiae* (*Adapted from Boiteux and Radicella, 1999*).

1. Unspecific binding of the repair enzyme to DNA.
2. Scanning of the DNA by the protein
3. Recognition of the abnormal structure caused by oxidative damage. (The unusual structure itself may help to form a DNA enzyme complex)
4. Displacement and flipping out of the nucleotide carrying the damaged base, by a mechanism which facilitates it's insertion into the active site pocket of the enzyme.
5. The C'1 position of the flipped out nucleotide is attacked nucleophilically by the activated amino group of the lysine residue of the repair enzyme.
6. The cleavage of the glycosylic bond between the damaged base and DNA resulting in the release of the damaged base and formation of a transient ENZYME –DNA intermediate.
7. Cleavage of the phosphodiester bond via a β elimination reaction.
8. Dissociation of the repair enzyme from the DNA.

The steps 4-8 above, are based on the crystal structure of endonuclease III and the uracil DNA glycosylases (Thayer *et al.*,1995; Savva *et al.*,1995; Mol *et al.*,1995). The mechanism by which the bases are flipped out by these enzymes is not well understood and the proposed scheme faces many ambiguities (Girard and Boiteux *et al.*,1997; Boiteux and Radicella, 1999).

1.3.2.4 Nucleotide excision repair (NER)

Nucleotide excision repair (Fig.1.12), is a general repair pathway identified long before the discovery of base excision repair. This universal repair mechanism removes a remarkably diverse array of structurally unrelated lesions from DNA. The most well known substrates for NER include various UV induced photoproducts, (CPDs and 6-4 PPs), chemical adducts and some types of cross links. Similar to BER,

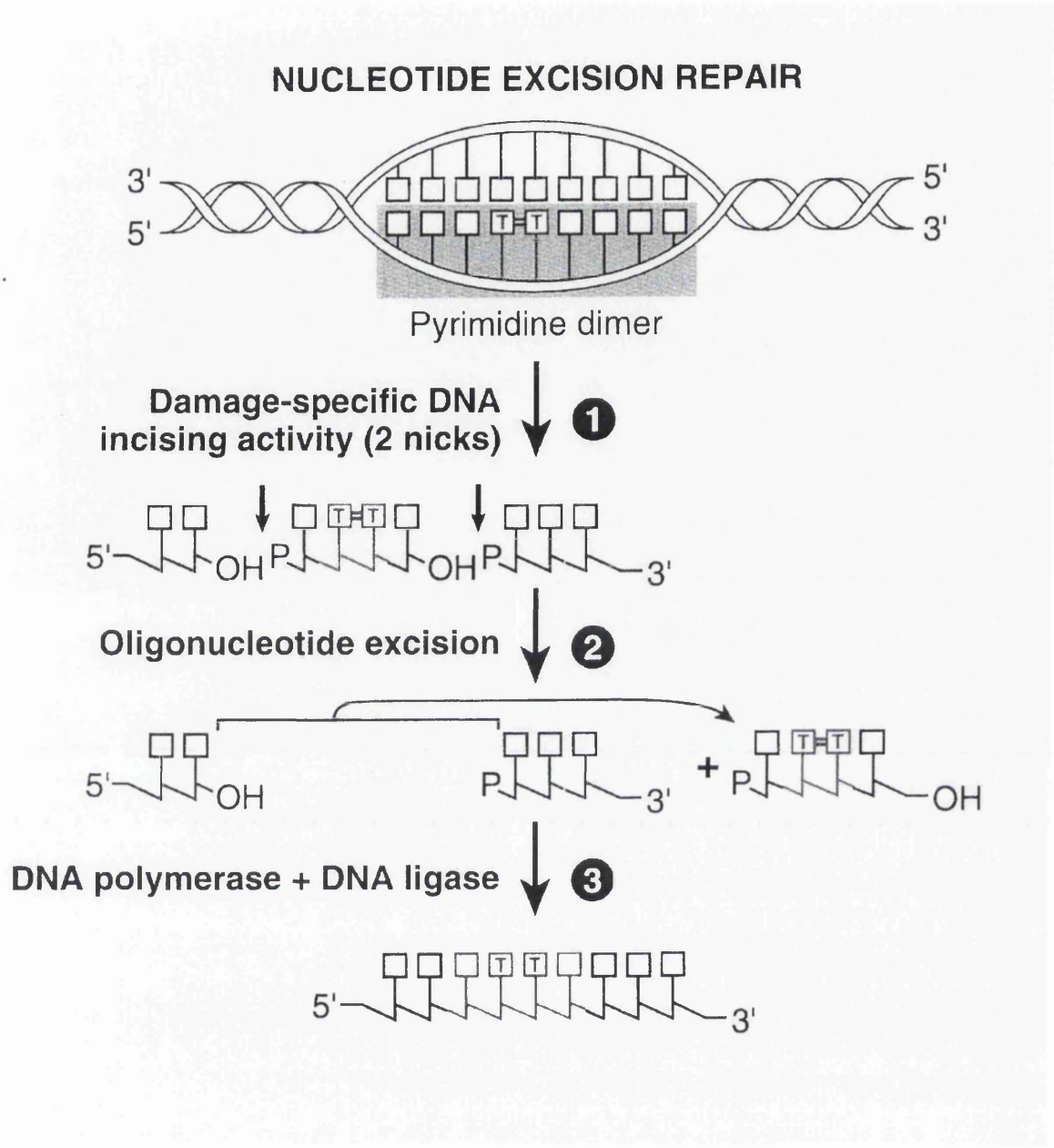


Figure 1.12 Schematic representation of basic process of nucleotide excision repair (*Reproduced from Friedberg et al., 1995*)

NER is a multiprocess mechanism involving the excision of the damaged nucleotide leaving a gap, which is then filled by DNA polymerases and rejoined at the 3' end by DNA ligases. However, NER is biochemically far more complicated and involves a far greater number of proteins. The mechanism of NER has been well studied in *E.coli* and more recently in eukaryotes both *in vitro* and vivo. NER in prokaryotes and eukaryotes consist of the same basic steps. These steps are a damage recognition, a dual incision both 5' and 3' to the lesion (1), excision of the oligonucleotide containing the lesion (2), replacement of the excised bases using the undamaged strand as template, ligation of the newly synthesised DNA to the existing DNA strand (3). Theoretically the reaction is error free (Friedberg *et al.*, 1995) (Fig. 1.12).

1.3.2.4.1.1 NER in *E.coli*

The UVR system is responsible for the NER in *E.coli*. The basic process requires 6 structural genes (*uvrA, uvrB, uvrC, uvrD, polA*, and DNA ligase) and two regulatory genes (*recA* and *lexA*). These genes have been cloned, sequenced and protein products have been purified to apparent homogeneity. The following scheme (Fig. 1.13) summarises the main steps of basic NER in *E.coli* (Friedberg *et al.*,1995; Sancar, 1996; Lehmann *et al.*, 1996; Moller and Wallin, 1998; Petit and Sancar, 1999).

1. The damage recognition process is initiated by UvrA and UvrB proteins. UvrA facilitates the loading of UvrB onto DNA lesion (Sancar,1996). A heterotrimeric A_2B_1 complex is formed by association of a UvrA dimer and a UvrB. The A_2B_1 complex then binds non-specifically to duplex DNA (Orren and Sancar, 1989).

2. Binding of A_2B_1 complex to DNA activates the ATPase/helicase activity of UvrB; enabling the complex to scan along the DNA and bind to the damaged sites. After the formation of a A_2B_1-DNA complex at damaged sites, ATP hydrolysis by UvrB gives rise to a local melting of 5bp around the lesion. This structural change allows extensive contacts between the UvrB and the damaged strand followed by dissociation of UvrA (Lin and Sancar, 1992).

3. UvrC then binds the preincision UvrB-DNA complex inducing a conformational change, which allows the UvrB protein to incise the DNA, 4 nucleotides 3' to the damage site. Following this 3' incision, the UvrC protein catalyses nicking of the DNA 7 nucleotides 5' to the lesion. Yielding an oligonucleotide of 12-13 nt (Lin and Sancar, 1992). the contribution of the two subunits to both 3' and 5' incisions has not been excluded (Sancar, 1996).

4. Following the bimodal incision, the product of *uvrD* gene, Helicase II binds to the incised DNA which allows the release of the oligonucleotide fragment and the UvrC subunit (Orren *et al.*, 1992).

5. Following the release of UvrC the 3'-OH at the 5' incision site becomes accessible to DNA polymerase I (the polA gene product) which fills in the gap and at the same time displaces uvrB. The remaining nick after repair is sealed by DNA ligase (Orren *et al.*, 1992). The role of NER in the repair of oxidative base damage will be discussed later.

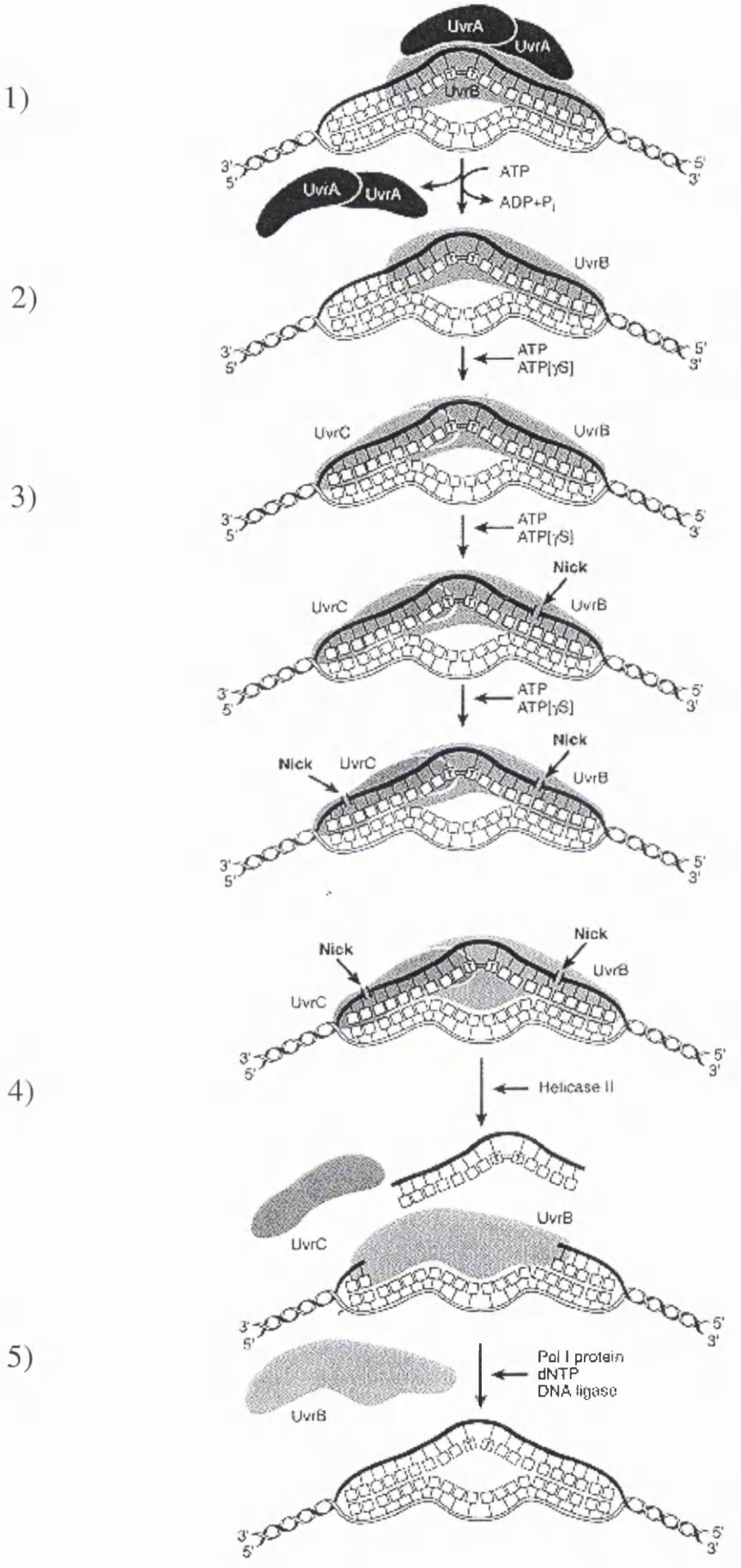


Figure 1.13 Diagramatic representation of basic process of NER in *E.coli* (*Adapted from Friedberg et al., 1995.*

Transcription coupled repair in *E.coli*

It is now established that NER has two sub-pathways for the removal of transcription blocking lesions. The relatively slow global genome repair for the removal of all transcription blocking lesions irrespective of their position in DNA, and the transcription–coupled repair pathway, which is responsible for the preferential/rapid removal of these lesions from the transcribed strands (Mullenders, 1998; Vrieling *et al.,* 1998).

The intimate link between NER and transcription was revealed during the last couple of decades. In 1985, Bohr *et al.* discovered that the repair of CPD's in active *DHFR* gene in a Chinese hamster ovary (CHO) was significantly faster than the average repair rate for the entire genome, which is essentially inactive. Based on these findings, it was suggested that the faster repair of an active gene is a direct result of relaxed chromatin structure provided by active transcription (Bohr *et al.,* 1985, Bohr and Hanawalt, 1987). Subsequently, it was shown that the transcribed strand of *DHFR* gene in CHO cells and the transcribed strand of the *lac* operon in *E.coli* is repaired considerably faster than the non-transcribed strand; suggesting that the rapid repair of transcriptionally active genes is partly due to the faster repair of the lesions in the transcribed strand by a process termed transcription coupled repair (TCR) (Mellon *et al.*, 1987; Mellon and Hanawalt, 1989). Based on these findings, it was suggested that *E.coli* RNA polymerase stalled by lesions in the template strand may act as a condensation site for the assembly of repair complexes. However it was demonstrated that the incision by Uvr (ABC) exinucleases was inhibited by the presence of a stalled transcription complex *in vitro* (Selby and Sancar, 1990). Later it

was shown that a transcription repair coupling factor (TRCF) encoded by the *mfd* gene in *E.coli* (Selby and Sancar, 1991), releases the stalled RNA polymerase by an ATP-dependent reaction. Since the stimulation of the repair of the transcribed strand by TRCF needs active transcription, the following model was proposed by Selby and Sancar, (1993): TRCF binds the stalled polymerase displacing it together with truncated RNA. Subsequently, an interaction between TRCF and UvrA in the A_2B_1 heterotrimer mediates the recruitment of NER enzymes to the transcription-blocking lesion (Selby and Sancar, 1993).

It has also been shown that the efficiency of TCR is influenced by the position of the damage relative to the transcription start site (Selby and Sancar, 1995). Using both an *in vitro* repair assay and an *in vivo* investigation of repair at nucleotide resolution in the *lacI* and *lacZ* genes, it has been demonstrated that enhanced repair of the transcribed strand starts respectively 15, 10 and 32 nucleotides downstream of the transcription start sites (Selby and Sancar,1995; Kunala and Brash, 1995). These results suggest that for TCR of the transcribed strand to take place, the RNA polymerase must have cleared the promoter and entered the elongation stage of polymerization (Selby and Sancar, 1995).

1.3.2.4.2 NER in *S.cerevisiae*-Rad3 epistasis groups

In terms of responses to DNA damaging agents, *S.cerevisiae* has been intensively studied. More than 30 loci have been characterised that confer resistance to killing by UV or ionising radiation. These loci fall into three large categories designated epistasis groups. Each epistasis group is believed to present one of three distinct

cellular responses to DNA damage (1.3). If two genes are involved in sequential steps of a single multistep biochemical pathway, those two genes are said to be in the same epistasis group. In other words, if mutations at two different genes confer a phenotype which is the same as the phenotype represented by each single mutation alone, the two genes are said to be epistatic with respect to one another. The three epistasis groups of *S.cerevisiae* are shown in the table 1.3. Among these the *RAD3* epistasis group is involved in NER and inactivation of any of these genes results in increased sensitivity to UV radiation or many chemicals including those that induce bulky adducts (Friedberg et al., 1995; Lehmann et al., 1996).

The members of *RAD3* epistasis group can themselves be categorised into two distinct subgroups. The first group that shows a very high sensitivity to UV light has genes essential for the incision step of NER. These include *rad 1,2,3,4,10,14* and *25*. The second group represents a moderate sensitivity to UV and includes *rad 7, 16, 19, 20, 21* and *22*. The second group are only partially defective in NER and it has been suggested that they only have an accessory role in NER processes *in vivo*. *rad23* is an exception and although it shows an intermediate sensitivity, appears to be totally NER defective (Reed *et al.*, 1996a; Verhage 1996b). Following sections discuss different steps of NER in *S.cerevisiae*.

Damage recognition –Rad 14 (Human XPA and Rpa)

The *RAD14* gene encodes a protein of 371 amino acids which presents a high degree of sequence homology to the human XPA protein (Guzder *et al.*,1995a). Rad14 protein is a zinc metalloprotein which binds specifically to DNA exposed to UV light but not to undamaged DNA. CPDs , 6-4PPs and endonuclease III sensitive sites are

RAD3 group	RAD52 group	RAD6 group
RAD1	***RAD50***	***RAD5(REV2)(SNM2)***
RAD2	***RAD51***	***RAD6***
RAD3	***RAD52***	***RAD8***
RAD4	***RAD53***	***RAD9***
RAD7	***RAD54***	***RAD15***
RAD10	***RAD55***	***RAD18***
RAD14	***RAD56***	***RADH***
SSL1	***RAD57***	***REV1***
SSL2(RAD25)	***RAD24***	***REV3(PSO1)***
TFB1	***XRS2***	***CDC9***
RAD16		***REV5***
RAD23		***REV6***
CDC8		***REV7***
CDC9		***CDC7***
MMS19		***CDC8***
PSO2(SNM1)		***MMS3***
PSO3		***PSO4***
UVS12		***UMR-1***

Table 1. 3 Epistasis groups of *S.cerevisiace* involved in cellular responses to DNA damage (*Adapted from Friedberg et al., 1995*)

not repaired in *rad14* deletion mutants (McCrready 1994; Reed *et al.*,1996b; Guzder *et al.*, 1993)

It has been demonstrated that XPA and Rpa proteins in human cells interact. This suggests that Rpa may also play a role in the recognition of DNA damage (He *et al.*,1995; Li *et al.*,1995; Burns *et al.*, 1996). Whether Rad14 and Rpa similarly interact in yeast, is a question yet to be answered but interaction between Rad14 and Rad23 has been shown in *S.cerevisiae* and it has been argued that this interaction initiates complex formation between Rad14 and other proteins essential for the subsequent stages of NER (Guzder *et al.*,1995a).

Unwinding the DNA at damage sites

Rad3 and Rad25 (Human XPD and XPB)

The helicase activities of Rad3 and Rad25^{SSL2} have been shown to be essential for NER *in vitro* (Sung *et al.*,1996). These data support the proposed model in which the joint action of Rad3 and Rad25 helicases unwind the DNA structure at damage sites yielding a bubble, approachable to the proteins responsible for the dual incision step of NER (Sung *et al.*,1996; Guzder *et al.*, 1994b). Rad3 has also been shown to have a DNA damage binding activity as well as its helicase activity (Sung *et al.*,1994).

Dual incisions/excision

Rad1 and Rad10 (human XPF^{ERCC4} and ERCC1)

The *RAD1*and *RAD10* genes are essential for the incision step of NER. The Rad1-

Rad10 complex is a heterodimer composed of 1 molecule of Rad1 and 1 molecule of Rad10 (Tomkinson *et al.*, 1994). This heterodimer has a single stranded endonuclease activity, while neither of the two proteins alone can catalyse the endonucleolytic degradation of ssDNA (Sung *et al.*,1993b; Tomkinson *et al.*,1993).

Local denaturation of DNA at damage sites (bubble formation) creates duplex/3'-single stranded junctions representing a substrate for the Rad1-Rad10 complex. The Rad1-Rad10 complex specifically acts on the junction between double stranded DNA and 3' single stranded tails and can incise DNA at the 5' side of the lesions during NER (Bardwell *et al.*,1994b) (Fig. 1.14).

Apparently there is an interaction between the DNA damage recognition protein Rad14 and the Rad1-Rad10 complex, suggesting a mechanism for targeting the Rad1-Rad10 complex to the damage site (Guzder *et al.*,1996b). A similar interaction has been observed in humans between the ERCC1-XPF^{ERCC4} complex with the XPA proteins (Park and Sancar, 1994). XPA, ERCC1 and XPF^{ERCC4} are the human homologues of the yeast Rad14, Rad10 and Rad1 proteins respectively.

Rad1 or Rad10 do not share any homology with UvrC, which is responsible for the incision on the 5' side of damaged DNA in *E.coli*, Nevertheless limited amino acid homology has been reported between UvrC and ERCC1 protein (Friedberg *et al.*,1995).

Rad2 (HUMAN XPG protein)

It has been demonstrated that the product of the *RAD2* gene has single stranded

endonuclease activity with the function to recognise the junction between duplex DNA and 5' single stranded tails i.e. incision on the 3' side of the DNA lesion, opposite to that of the Rad1-Rad10 complex. (Habraken *et al.*,1995) (Fig. 1.14).

The human homologue of Rad2, the XPG protein has been shown to cleave specifically at duplex/5'ssDNA (O'Donovan *et al.*,1994a). The *rad2*, *rad1*, or *rad10* mutants have no ability to incise damaged DNA suggesting that unlike in *E.coli*, bimodal incision during yeast NER is a concerted reaction (Guzder *et al.*,1995b).

The Rad1-Rad10-Rad14 complex: NEF1

Rad1-Rad10 and Rad14 are suggested to form a protein complex known as NEF1 (Guzder *et al.*, 1996b).

Rad4 and Rad23 : NEF2

The *RAD4* and *RAD23* gene products have been shown to copurify, suggesting the existence of a Rad4-Rad23 protein complex *in vivo* (Guzder *et al.*,1995b). This complex has been suggested to constitute a second NER factor (NEF2). In a reconstituted nucleotide excision repair reaction, incision of UV damaged DNA is dependent on NEF2 indicating its role in an early step of the repair process (Guzder *et al.*,1996b). Rad23 apparently has no catalytic activity but is suggested to be involved in mediation of the assembly of a functional NER complex (Guzder *et al.*,1995a). It has been demonstrated that NEF2 binds specifically to UV-damaged DNA. However, NEF2 does not possess an enzymatic activity and its function in the damage specific incision reaction remains to be further investigated (Guzder *et al.*, 1998a).

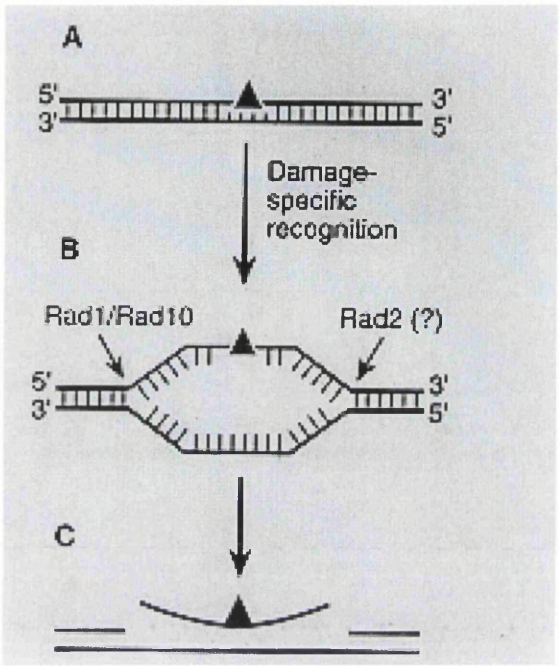


Figure 1.14. A model for bimodal incision of DNA lesion during NER in *S.cerevisiae*. After damage recognition (A), a bubble is formed at the site of base damage(B). The Rad1/Rad10 endonuclese recognizes and incises the duplex/3'-single strand junction in the bubble 5' to the base damage, while Rad2 probably recognizes and incises the duplex/5'-single strand junction 3' to the base damage. The oligonucleotide produced by this bimodal incision is subsequently excised (*Adapted from Friedberg et al., 1995*).

General transcription factor TFIIH

A substantial number of the proteins involved in DNA repair pathways have second functions (Reviewed in Lehmann, 1998). It has been shown in both yeast and mammalian cells that a number of NER genes are subunits of the general transcription factor TFIIH (Feaver *et al.*, 1993; Wang *et al.*, 1994; Sung *et al.*, 1996; Schaeffer *et al.,* 1993; Drapkin *et al.*, 1994). Mutations in genes encoding the subunits of TFIIH, give rise to cancer prone DNA repair disorder Xeroderma pigmentosum or the noncancer prone multisystem disease Trichothiodystrophy, the phenotypes of which may be associated with deficiency in transcription (Lehmann and Norris, 1989; Cleaver and Haltner, 1995; Guzder *et al.*, 1995c Lehmann, 1998). Different forms of TFIIH have been isolated, which are transcription or NER competent (Svejstrup *et al.*, 1995; Sung *et al.*, 1996). The yeast TFIIH is composed of six subunits including Rad3 and Rad25, which both possess an ATP dependent DNA helicase activity (Guzder *et al.*, 1994; Sung *et al.*, 1987), and four other subunits, TFB1, SSL1, p55 and p38 (Feaver *et al.*, 1993; Guzder *et al.*, 1995b). In addition to TFIIH, a subassembly of the factor that doesn't contain Rad3 and Rad25 have been purified designated TFIIH–incomplete or TFIIHi. It has been demonstrated that neither TFIIHi nor a mixture of purified Rad3 and Rad25 proteins alone promote the incision of UV damaged DNA, indicating that Rad3, Rad25 and one or more subunits of TFIIHi are absolutely required for incision step of NER (Sung *et al.*, 1996). It has also been shown that TFIIH alone is not sufficient for transcription by RNA polymerase II *in vitro* and it can only promote transcription when it is in a complex with another protein TFIIK (Svejstrup *et al.,* 1995). TFIIK is inessential for the incision step of NER and it's association with TFIIH has functional relevance only for Pol II transcription (Sung *et*

al., 1996).

TFIIH-Rad2 complex- NEF_3

It has been demonstrated that Rad2 exist in a stable complex with TFIIH . This complex is designated NEF_3 (Habraken *et al.*, 1996).

A proposed model for NER in *S.cerevisiae*

Biochemical fractionation of the yeast cell extracts and reconstitution of NER *in vitro* using the purified proteins, has suggested that NER is mediated by the sequential assembly of repair factors and not by a pre-assembled repairosome. These studies has shown that the Rad1-Rad10 complex, the Rad2 protein, the Rad4-Rad23 complex, Rad14 protein, replication protein A or RPA, and the RNA polymerase II transcription factor TFIIH, are essential for the incision reaction of damaged DNA *in vitro* and no incision reaction can occur if any of these proteins are absent (Guzder *et al.*, 1996b). It is suggested that these proteins are organised into different functional subassemblies called nucleotide excision repair factors or NEFs. Based on this idea, a model has been proposed by Habraken *et al.*,(1996) for NER in yeast which involves the following steps (Fig. 1.15). These process have been recently reviewed by Araújo and Wood, (1999) (Fig. 1.16).

1. NEF1 (Rad1, Rad10 and Rad14) and RPA bind to the damage site because of the high affinities of RPA and Rad14 for UV damaged DNA.(Guzder *et al.*, 1993; Burns *et al.*, 1996).
2. Following the binding of Damaged DNA to NEF1 and RPA, NEF2 (Rad4 and Rad23 complex) is brought to the damage site via the interaction of Rad23 with

Rad14 in NEF1 (Guzder *et al.*, 1995a). Subsequently, by the interaction of TFIIH with the Rad23 component of NEF2, and also via the affinity of Rad3 for damaged DNA, (Sung *et al.*, 1994), NEF3 (TFIIH and Rad2)is incorporated into the protein assembly. The possible interaction of Rad2 in NEF3 with RPA, can also aid the assembly formation.

3. The ATP dependent helicase activities of Rad3 and Rad25 in NEF3, unwinds the DNA at the damage site (Sung *et al.,* 1996).

4. Rad1-Rad10 and Rad2 nucleases then initiate the 5' and 3' incisions of the damaged DNA (Habraken et al., 1993; Habraken, 1994b; Habraken *et al.,* 1995; Bardwell *et al.*, 1994b; Matsunaga *et al.*, 1996).

Rad7-Rad16 complex-NEF4

The *RAD7* and *RAD16* encoded proteins, which are responsible for the repair of transcriptionally inactive DNA, have been shown to exist as a stoichiometric complex designated NEF4. Recently it was shown that NEF4 has a high affinity for UV damaged DNA and when it is added to the reconstituted NER system consisting of NEF1,NEF2,NEF3 and RPA, gives rise to significant increase in the damage specific incision capacity of NER (Guzder *et al.*, 1997). Furthermore, it has been shown that NEF4 possesses an ATPase activity. The free energy released from ATP hydrolysis fuels the translocation of NEF4 in scanning for DNA damage. Binding of NEF4 to the lesions give rise to a down regulation of ATP hydrolysis and a DNA damage-NEF4 complex is stabilised which serves as a nucleation site for the assemblage of other NER factors (Guzder *et al.*, 1998b).

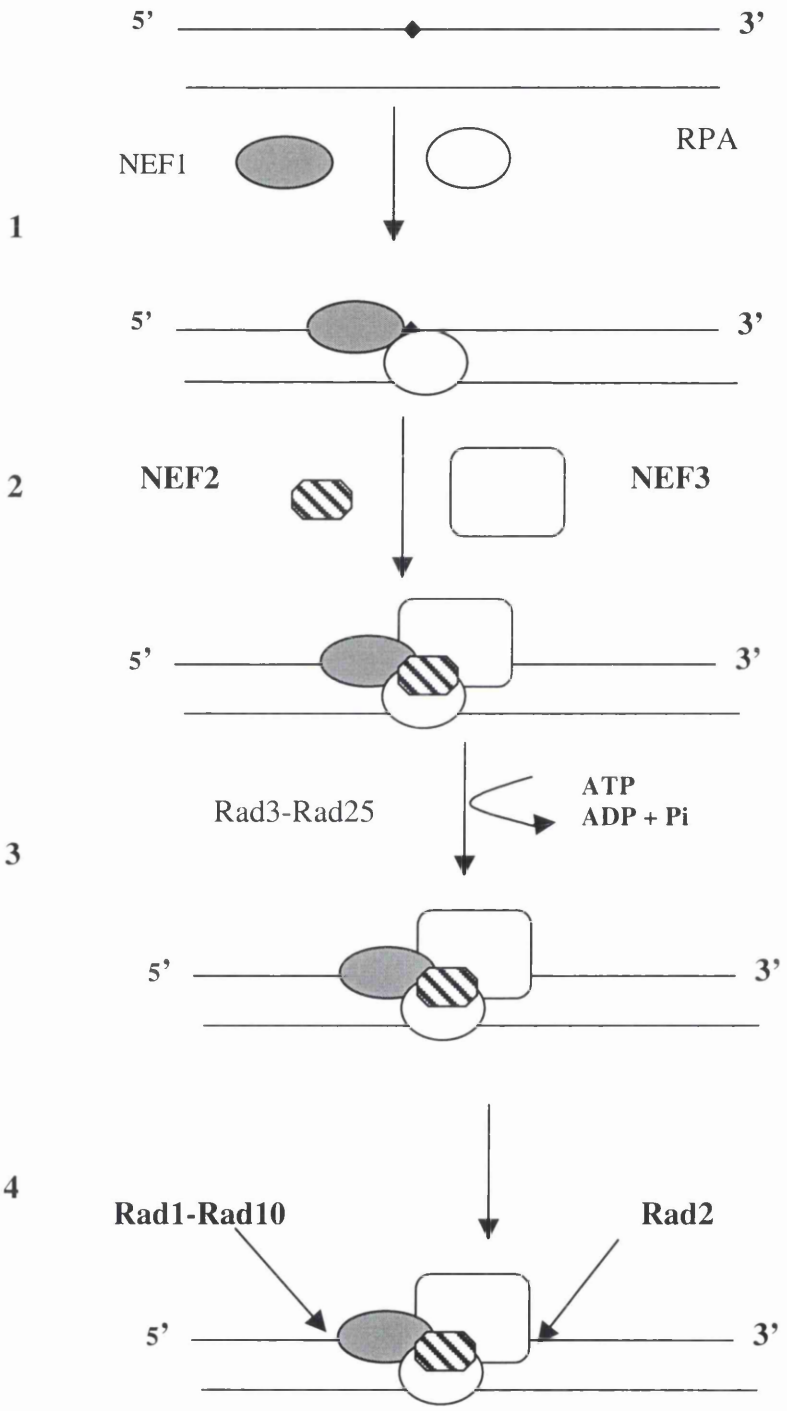
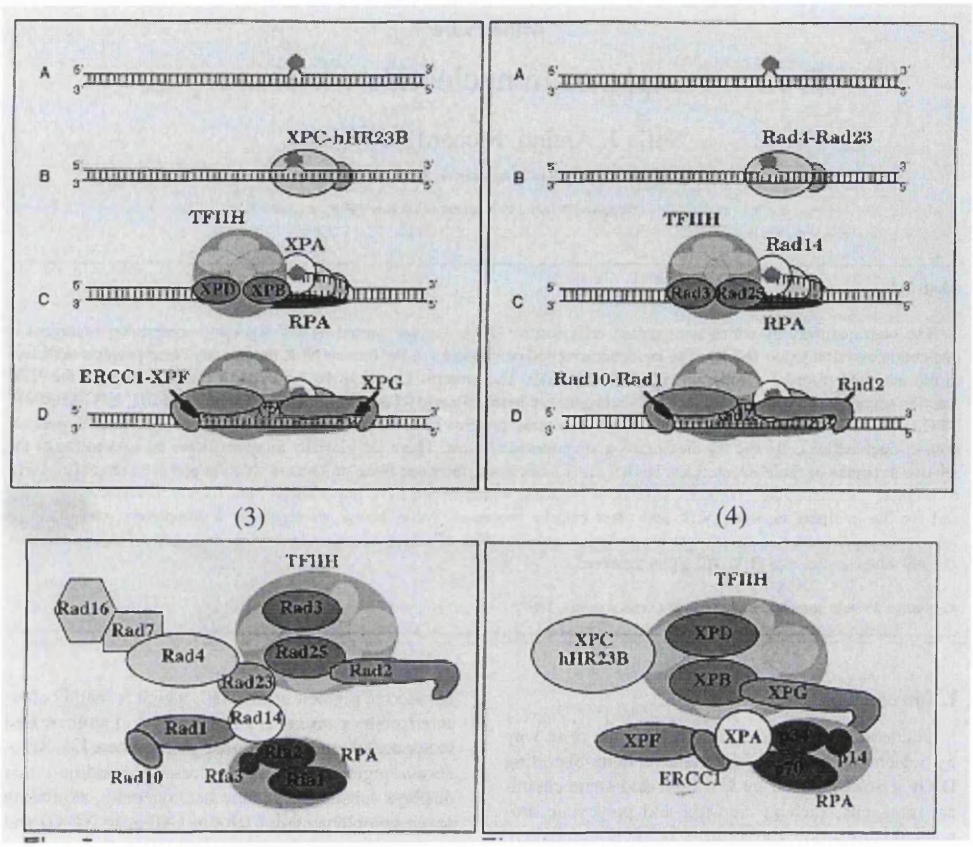


Figure 1.15 Sequential assembly of NER factors at the damage sites (*Adapted from Habraken et al., 1996*)

(1) (2)



(3) (4)

Figure 1.16 (1) and (2) A model for dual incision reaction of nucleotide excision repair in Human cells and *S.cerevisiae* respectively. (A: Helix distorting lesion, B: Recognition of damage, C: Unwinding the lesion-containing region, D: Dual incision.

(3) Protein –Protein interactions between the proteins involved in dual incision function of NER in S.cerevisiae.

(4) Protein – Protein interactions between the proteins involved in dual incision function of NER in humans.

(Adapted from Araújo and Wood, 1999)

Transcription-coupled and global genome repair in *S.cerevisiae*

At present no defined *in vitro* system for eukaryotic TCR has been developed and it appears that the molecular basis of TCR in eukaryotes is far more complex than that of *E.coli*. It has been demonstrated that TCR in yeast is dependent on transcription by RNA polymerase II (Leadon and Lawrence, 1992; Sweder and Hanawalt, 1993). The discovery of the dual role of basal transcription factor TFIIH in NER and in initiation of transcription by RNA polymerase II is considered strong evidence for direct transcription–repair coupling (Feaver *et al*, 1993). The obligatory loading of TFIIH onto promoter sites during transcription initiation, could provide a clear scenario for a direct coupling of RNA polymerase II transcription and NER. However, using an *in vitro* assay, it was shown that although TFIIH remains associated with the transcription complex during the first steps of nascent mRNA synthesis, it is dissociated from the transcription machinery between positions +30 and +68, and no TFIIH was present in isolated stalled transcription elongation complexes (Zawel *et al.*, 1995; Goodrich and Tjian, 1994). This finding that TFIIH is not part of the elongation complex, suggested that additional components are necessary for recruitment of TFIIH at the DNA damage sites where RNA polymerase II stalls. In humans, the possible candidates for this function are thought to be the Cockayne syndrome (CS) group A and B gene products (Henning *et al.*, 1995; Troelstra *et al.*, 1992); as the preferential repair of transcribed strand is absent in cells from CS patients (Van Hoffen *et al.*, 1993). *S.cerevisiae* homologues of *CSA* and CSB, the *RAD28* and *RAD26* genes respectively, have been cloned (Bhatia *et al.*, 1996; Van Gool *et al.*, 1994). It was shown that Rad26 has a DNA-dependent ATPase activity (Guzder *et al.*, 1996a). In the *rad26* mutants, the repair rate of the *RPB2* transcribed

strand had been shown to be reduced to the repair rate of the nontranscribed strand, supporting the requirement of *RAD26* for TCR in yeast (Van Gool *et al.*, 1994). However, by using high resolution repair mapping, it was demonstrated that although TCR of CPDs is severely reduced in *rad26* mutants, the lesions located in a small region immediately downstream of transcription initiation are efficiently removed. This transition from fast to slow repair in the transcribed strand of *RPB2* is in perfect agreement with the previously identified region where TFIIH is released from the RNA polymerase II complex *in vitro* (Tijsterman *et al.*, 1997). Contrastingly, TCR of the *MFA2* gene operates equally on CPDs throughout the strand, and there was no evidence for more rapid repair in the early parts of the transcript (Teng, personal communication). Thus the role of TFIIH at present, is by no means clear.

The yeast *RAD7* and *RAD16* genes are essential for the repair of nontranscribed strands or global genome repair (Verhage *et al.*, 1994). Previously it was demonstrated that CPDs are not removed from the inactive HMLα locus in *rad7/16* mutants. Considering that the HMLα locus has a suppressed chromatin structure, these results suggested that the Rad7 and Rad16 are involved in the opening of folded chromatin regions making them approachable for the NER complex (Terleth *et al.*,1990). However, the NER of lesions other than CPDs can proceed in HMLα, thus questioning this interpretation (Reed *et al.*, 1996a). Recently, examining the repair at the level of nucleotide in the *MFA2* gene of *S.cerevisiae* has demonstrated that *RAD16* is required to repair the NTS except for the upstream regulatory region where repair albeit slower can proceed (Teng *et al.*, 1997).

It has been reported that the *rad7/16 rad26* double mutants retained the ability to

remove CPDs from the transcribed strand of the *RPB2* gene although in a reduced rate. This suggested the existence of a *RAD26*- independent residual TCR via some other gene products (Verhage *et al.*, 1996a). The *RAD28* gene appeared to be completely dispensable for TCR in yeast (Bhatia *et al.*, 1996), and some other likely candidates such as transcription elongation factor S-II were ruled out as responsible for the Rad26-independent TCR. These observations supported the scenario that the stalled RNA polymerase II at a lesion in the transcribed strand, forms an assembly site for NER complex (Verhage *et al.*, 1997) and some other factors may enhance these process without direct involvement in coupling of NER to transcription. For instance, the TFIIH complex is essential for NER of both strands (Sweder and Hanawalt, 1994; Wang *et al.*, 1994; Sweder *et al.*, 1996). Hence this complex is not directly involved in transcription–NER coupling in eukaryotes. However, assuming that this complex has an affinity for a stalled transcription elongation complex, it could enhance the rate of recruitment of the NER machinery at such sites (Verhage *et al.*, 1997). It has been reported that some mismatch repair proteins are also involved in TCR of thymine glycol in *S.cerevisiae* (Leadon, 1998).

More recently, the result of some experiments in *S.cerevisiae* have demonstrated that the yeast RNA polymearse II transcription *in vitro* is inhibited in the presence of NER. This inhibition was strictly dependent on active NER and was shown to be complemented with purified holo-TFIIH. It was also shown that this inhibition required the *RAD26* gene. These results suggested that in the presence of active NER, TFIIH is preferentially mobilised from the basal transcription machinery to serve in NER (You_ZY *et al.*, 1998).

The present knowledge of TCR in eukaryotic cells have been further complicated by the result of experiments investigating the repair of CPDs at nucleotide level in the human *JUN* gene (Tu *et al.*, 1996) and the yeast *MFA2* gene (Teng *et al.*, 1997). These studies reported that efficient TCR begins upstream of the transcription start site, ruling out that elongation is necessary for NER. In addition, It was observed that repair in both strands of the *JUN* gene was more efficient near the transcription start site relative to the regions further downstream. Based on these findings, it was argued that a higher concentration of TFIIH complexes within the promoter region of the *JUN* gene is the cause of the faster repair (Tu *et al.*, 1996). However, a different trend was observed for the yeast *MFA2* gene, where repair was most efficient within the coding sequence (Teng *et al.*, 1997).

1.3.3 Other DNA repair mechanisms

Mismatch repair

Mismatch repair is one of the most important excision repair mechanism which has an important role in reducing error rate in cellular DNA replication or recombination (Modrich, 1991; Modrich and Lahue, 1996). Several distinct mismatch repair pathways have been identified in both prokaryotes and eukaryotes. Infidelity during DNA replication or recombination processes, and the spontaneous base damages such as deamination of cytosine to create uracil in DNA, are responsible for a significant proportion of mismatched base pairs in DNA (Friedberg *et al.*, 1988). When the mismatches are created during replication, the erroneous base of the mispair will be located in the newly synthesised daughter strand and the correct base in the parental

DNA strand. Because the two bases are perfectly normal, the repair mechanism requires a sign to identify the wrong base that has to be removed and corrected. In *E.coli* due to the postsynthetic methylation of adenine at GATC sites, catalysed by Dam methylase, the GATC sequences are methylated in parental strand and not in newly synthesised daughter strand. This pattern allows the mismatch repair pathway to distinguish the newly synthesised strand carrying the wrong base from the parental strand which has the correct base. This mechanism is called methyl directed mismatch repair and it is the most understood general mismatch repair system (Modrich, 1991). The methyl directed mismatch repair system of *E.coli* involves three key enzymes, MutH, MutL and MutS (Kolodner 1995; Modrich and Lahue 1996).

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The mismatch repair of eukaryotes is fundamentally different from methyl directed mismatch repair mechanisms in *E.coli*. The strand specification in eukaryotes, does not involve DNA methylation. It has been shown that DNA is not significantly methylated in *S.cerevisiae* (Kolodner,1996; Modrich and Lahue, 1996; Kolodner and Marsischky, 1999). Deficiency in correction of mismatches in *S.cerevisiae* results in an elevated frequency of post meiotic segregation (PMS). A number of mutants with high PMS frequencies have been isolated in *S.cerevisiae* (Williamson *et al.*,1985). Cloning and sequencing of some of these mutants has revealed three MutL homologues, PMS1 (Kramer *et al.*,1989), MLH1 and MLH2 (Prolla *et al.*,1994 a). The function of MLH2 is not yet clear but it has recently been shown that Pms1 and Mlh1 proteins physically interact to achieve their biological function (Prolla *et al.*,1994 b). At least six homologues of MutS designated MSH1-MSH6 have been characterised in *S.cerevisiae* (Kolodner *et al.*,1996). Genetic and biochemical analysis suggests that three of these homologues MSH2, 3, and 6 are involved in mismatch

recognition (Marsischky *et al.*,1996). The exact mechanism of the mismatch repair in *S.cerevisiae* remains unclear. Human homologues of MutL and MutS have been identified (reviewed in Kolondner, 1996).

Important interactions have been reported between mismatch repair and other repair pathways, notably nucleotide excision repair. In human and bacterial cells, deficiency in mismatch repair has shown to attenuate transcription coupled repair (Modrich and Lahue, 1996). The inheritance of mutations in mismatch repair genes is linked to hereditary nonpolyposis colon cancer (HNPCC), one of the most common inherited cancer susceptibility syndromes known in humans (Papadopoulos *et al.*,1994).

Physical interactions have been demonstrated between components of mismatch repair and NER in *S.cerevisiae,* suggesting that MSH2 and possibly other components of DNA mismatch repair exist in a complex with NER proteins and they might be operating in common processes (Bertrand *et al.*, 1998). There is also evidence suggesting the involvement of mismatch repair proteins in the transcription coupled repair of thymine glycols in *S.cerevisiae* (Leadon *et al.*, 1998).

It should be noted that the mismatch repair mechanism, unlike BER and NER, does not recognise chemically modified nucleotides or bases. Rather, the mismatch repair enzymes recognise the normal nucleotides within DNA.

The *RAD52* epistasis group-Recombinational repair in *S.cerevisiae*

Recombination is involved in the removal of single or double strand breaks. Double strand breaks are potentially lethal lesions since there is no undamaged

complementary strand available for the excision repair mechanisms to use as a template. DSBs can be produced directly by the exposure of cells to ionising radiation or indirectly following the replication or repair of damaged templates. Mitotic recombinational repair mechanisms are the only known pathways to tackle DNA double strand breaks induced by ionising radiation. In *S.cerevisiae*, such damage is repaired principally by homologous recombination using the DNA sequence of the sister chromatid or homologue as the template in an error free manner (Szostak *et al.*, 1983). Other pathways of DSB repair have been characterised which are intrinsically error prone (Clever *et al.*, 1997). It should be noted that recombination repair may constitute a damage tolerance rather than a damage removal mechanism for some types of lesions. Such recombination bypass of damage, allows the progression of meiosis and presumably leaves the damage to be removed by other repair pathways (Swanson *et al.*, 1999).

In *S.cerevisiae*, the *RAD52* epistasis group contains most of the genes essential for homologous recombination. Mutations of the *RAD52* group are defective in meiotic and mitotic recombination and show extreme sensitivity to ionising radiation or other double/single strand break inducing agents, as well as sensitivity to UV radiation (Reviewed in Game, 1993; Friedberg *et al.*, 1995; Shinohara and Ogawa, 1995). *RAD51*, *RAD52* and *RAD54* have been identified as the most crucial members according to the severity of the mutant phenotypes (Game, 1993). The *RAD51* gene encodes a protein that has a sequence similarity to the bacterial recombination protein, RecA. Like RecA of *E.coli*, this protein forms protein-DNA filaments that can act as a scaffold to facilitate DNA pairing and strand exchange during homologous recombination (Clever *et al.*, 1997). In *S.cerevisiae*, the recombination rates have been shown to increase upon exposure of the cells to H_2O_2, suggesting a role for

recombination in the repair of oxidative base damage in yeast (Brennan et al., 1994).

The *RAD6* epistasis group -Postreplication repair or Translesion synthesis in *S.cerevisiae*

The concept of postreplication repair or translesion synthesis, was first proposed as a type of cellular response to tolerate DNA damage in *E.coli.* In the proposed model, the replication blocking lesion is not removed, but it is the gap opposite the lesion in the daughter strand which is repaired. Therefore, postreplication repair is sometimes referred to as daughter strand gap repair. (Hanawalt *et al.*, 1979).

In eukaryotes similar mechanisms have been identified. In *S.cerevisiae* genes in the *RAD6* epistasis group are believed to mediate postreplication repair. There is evidences for the existence of both error prone and error free mechanisms of postreplication repair and many lines of evidence indicate the role of *RAD6* and *RAD18* in both processes (McDonald *et al.*, 1997).

To resolve the replication block by the presence of gaps, Rad6 is targeted to the stalled replication machinery via its interaction with the single stranded DNA binding protein Rad18. The error prone pathway is then mediated by DNA polymerase ζ consisting of the Rev3 and Rev7 proteins, encoded by the *REV3* and *REV7* genes of the *RAD6* epistasis group. This polymerase is able to bypasses several types of DNA lesions, including CPDs, and AP sites. Such translesion bypass of damage enables the cells to proceed through the cell cycle, but it gives rise to increase in mutation rate (Broomfield et al., 1998).

Due to the limited number of postreplication repair mutants the exact mechanism of error free postreplication mechanism is not well understood (Broomfield *et al.*, 1998).

1.3.4 Substrate specificities of NER and BER

The majority of DNA lesions are repaired by either of the two excision repair mechanisms, BER and NER. BER enzymes recognise and repair few but very frequently occurring DNA lesions. For a damage to be removed by BER, there must be a DNA glycosylase that can specifically recognise that particular damage and considering the huge variety of DNA damages and limited number of damage specific DNA glycosylases, BER alone is not a sufficient mechanism to deal with the numerous damages imposed on DNA. This explains the vital necessity for a more general and flexible repair system with a much broader substrate specificity. NER is a more complex repair pathway employing many more proteins than BER. The NER enzymes can recognise damaged regions based on the abnormal structure that has been created as a result of the damage rather than the specific damage itself. Therefore, NER is more efficient in removing damages that change the DNA conformation. In contrast, most of the DNA damages recognised by BER, are not helix distorting lesions. Therefore, the general assumption is that BER mainly recognises the lesions that do not significantly change the normal B configuration of DNA. Until almost a decade ago, it was thought that NER can only recognise bulky helix distorting lesions (Moller and Wallin, 1998).

1.4 The present study -The Contribution of NER to the

repair of oxidative base damages

DNA oxidative base damages create only minor distortions to the DNA double helix (Kow *et al.*, 1989). Until very recently, for the reasons discussed above, the research on repair of oxidative DNA lesions mainly focused on the role of BER mechanisms; it was assumed that NER had no role in the repair of oxidative base damages (Moller and Wallin, 1998). However, several lines of evidence in prokaryotes and eukaryotes disprove this assumption. The involvement of NER proteins in the repair of oxidative base damages has been demonstrated in both TCR and global NER.

The involvement of NER proteins in transcription coupled repair of oxidative base damage

Thirty proteins have been shown to be involved in the process of NER in humans (Friedberg, 1995; Abousseckhara, 1995). Deficiency in these proteins can give rise to rare diseases such as Xeroderma pigmentosum (XP) and Cockayne syndrome (CS). XP is an autosomal recessive disease, which usually reflects a deficiency in early stages of NER, due to mutations in any of the seven different genes XPA-XPG, belonging to XP complementation groups A-G. Clinical phenotypes of XP include acute sun sensitivity, abnormal skin changes, high incidence of cancers in sun-exposed areas, and frequently progressive neurological degeneration. CS patients do not have predisposition to cancer but they exhibit sun sensitivity, severe growth defects, neuronal demyelination, mental retardation, microcephaly, skeletal and retinal abnormalities, and dental caries. In normal human cells, DNA damage due to UV light is preferentially removed from active genes by NER in a TCR process that

requires the gene products defective in Cockayne syndrome. More than 140 CS cases have been reviewed (Nance *et al.*, 1992) that are categorised into 5 complementation groups. Most of the CS patients exhibit only CS symptoms and fall into groups CSA and CSB. The hallmark of the cells from CS patients is the absence of TCR (Mellon *et al.*, 1987; Van Hoffen *et al.,* 1993; Leadon and Cooper, 1993).

It has been previously reported that the damage produced by ionising radiation which shows little in common with UV damage in the spectrum of lesions induced, can also be preferentially removed from the active genes in normal human fibroblasts and those taken from XPA patients, but that CSA and CSB mutant cells are defective in this process. This suggested that the generality of TCR extends to damages other than bulky lesions recognised by NER, and that a BER pathway might also be coupled to transcription, raising the possibility that defective TCR of oxidative damage contributes to the developmental defects associated with CS (Leadon and Cooper, 1993).

XP and CS are usually clinically and genetically distinct. However, in very rare cases, some CS patients have been assigned to XP complementation groups B, D, or G. Some of these XP/CS patients also exhibit an XP phenotype. The XPB (Weeda *et al.*, 1990b) and XPD (Weber *et al.*, 1990; Flejter *et al.*, 1992) gene products are DNA helicases with DNA dependent ATPase activity and are components of TFIIH. In contrast, no role in transcription has been identified for the XPG gene product (Scherly *et al.*, 1993; Shiomi *et al.*, 1994). XPG encodes a structure specific endonuclease that nicks damaged DNA 3' to the lesion in an early step of NER (O'Donovan, 1994).

In order to examine a possible link between TCR of ionising radiation damage, CS and NER functions, TCR has been measured in X-ray irradiated XP-G mutant strains from patients with and without clinical symptoms of CS. Cells from three XPG/CS patients were unable to preferentially remove thymine glycols from the transcribed strands of active genes, while TCR of this lesion was normal in cells from XPG patients with no CS. A striking common pattern was observed for three XPG/CS patients. The three patients analysed exhibited severely early–onset CS, but had few XP characteristics, possibly because of limited sun exposure and limited life span, as they all died in infancy or early childhood. All three patients had mutations that would lead to production of severely truncated XPG proteins. In contrast, the two sibling XPG patients with a very mild XP phenotype and no CS, are able to produce full length XPG but contain a missense mutation that interrupts it's function in NER (Cooper *et al.*, 1997; Nouspikel *et al.,* 1997).

These results suggested that the clinical manifestation of CS in XPG/CS patients is not linked to the incision role of XPG in NER. Instead, it was proposed that the XPG/CS phenotype is a consequence of disruption of protein-protein or protein-DNA interactions that are important for a second XPG function, its role in TCR of oxidative damage. These results also imply that TCR of oxidative damage is independent of the XPG incision function in NER, and even of NER as a whole. It is suggested that this activity is initiated by glycosylases and AP endonucleases in BER, and that XPG has an assembly function in a BER pathway similar to its role in establishing the pre-incision complex in NER (Nouspikel *et al.*, 1997).

Furthermore, XPG/CS patients also exhibit a decreased rate of removal of oxidative

damage from the genome as a whole, which probably contributes to the unusually severe clinical phenotypes of XP/CS patients. This may suggest an additional role for XPG in BER that is not coupled to transcription (Cooper *et al.*, 1997).

In *S.cerevisiae*, the TCR of H_2O_2 -induced thymine glycols from the *GAL7* gene has been investigated in two NER deficient mutants *rad1*Δ and *rad2*Δ. TCR was shown to take place at a normal level in both the wild type and the *rad1*Δ mutants. In contrast, in *rad2* the initial rate of repair of thymine glycol on the transcribed strand was significantly slower than that of the wild type cells with kinetics similar to the repair of non-transcribed strand. These results suggested that *RAD2* which is the *S.cerevisiae* homologue of XPG, facilitates transcription coupled BER of oxidative DNA damage in yeast via a mechanism that is distinct from its role in NER (Habraken *et al.*, 1994c; Leadon *et al.*, 1995).

The involvement of NER proteins in the global repair of oxidative base damage

The UvrABC complex in *E.coli* had previously been shown to recognise a wide range of DNA damages most of them being bulky adducts such as UV induced CPDs, which considerably distort the double helix (Sancar and Sancar, 1988). However, biochemical and genetic evidence shows that the UvrABC complex is also able to recognise a number of lesser distorting lesions, including O6 methyl guanine (Van Houten and Sancar, 1987), and aminofluorene-guanine adducts (Pierce *et al*,. 1989).

Further research indicated that the UvrABC complex can also recognise thymine

glycol and reduced AP sites (Kow *et al*,. 1990), and that UvrABC has the same incision activity on plasmids which contained thymine glycol versus pyrimidine dimer lesions (Moller and Wallin, 1998). These data suggest that UvrABC may well detect relatively minor differences in the perturbation of the DNA structure produced by oxidative base damage (Kung and Bolton, 1997; Kow *et al.*, 1990; Kung and Bolton, 1997).

To investigate the repair of oxidative damage *in vitro*, a number of singlet oxygen generating photosensitizers have been used. Among these, methylene blue plus visible light (MB- light) has been shown to induce DNA base damages mainly at guanine bases that can be recognised and incised by Fpg (Muller *et al.,* 1990). HPLC studies have shown that the major lesion induced by methylene blue is 8-oxoG. MB-light can also generate single strand breaks at a 17 fold lower frequency (Schneider *et al.,*1990). Transfection of MB-light treated phage M13 DNA into SOS induced *E.coli* cells has resulted in an elevated increase in GC to CG transversions, suggesting that MB-light can also induce other uncharacterised purine base alterations (McBridge *et al.,* 1992). *In vivo* and *in vitro* studies indicate that the UvrABC complex also recognises lesions produced by MB-light (Czeczot *et al.,* 1991). It has been reported that the transforming efficiency of pBR322 DNA treated with MB-light *in vitro* has a decreased efficiently in a strain of *E.coli* defective in both UvrA and Fpg, while survival was unaffected in either single mutant compared to an isogenic wild type strain. This suggested that Fpg and NER proteins in *E.coli* both have a role in the removal of oxidative base lesions and they can complement each other. In support of this conclusion, the *in vitro* studies show that the incision of pBR322 DNA treated with MB-light, takes place efficiently by both Fpg and the UvrABC complex

(Czeczot *et al.*,1991).

The involvement of NER in the repair of oxidative base damage has also been indicated in human cells. It has been shown that the XPA (human homologue of *RAD14*) gene product, which binds preferentially to DNA containing bulky adducts such as cisplatin and pyrimidine dimers, can also bind to DNA containing thymine glycol induced by osmium tetroxide (Moller and Wallin, 1998; Asahina *et al.*,1994) and to DNA containing 8-oxoG (Klein *et al.*,1992). By using purified human NER proteins essential for the removal of thymine dimers *in vitro* (XPA, RPA, TFIIH, including XPB and XPD, XPC, HHR23B, XPG and XPF/ERCC1) (Mu *et al.*, 1995; 1996), it has been shown that thymine glycol, 8-oxoG, urea and thymine dimer lesions cannot be excised from DNA if any of these proteins are absent (Reardon *et al.*, 1997; Moller and Wallin, 1998).

In *S.cerevisiae*, the direct contribution of NER to the repair of oxidative base damages is not well documented (Girard and Boiteux, 1997) but increasing evidence supports a role for NER in the repair of oxidative base damages in *S.cerevisiae.* These data will be discussed in detail in chapters 3 and 4 of this thesis.

The aim of the work presented in this thesis is to further investigate the contribution of NER to the repair of oxidative base damage in *S.cerevisiae.* In chapter 2, the *RAD14* gene was disrupted in either a wild type or an *ogg1* background to create strains defective in NER, BER or in both. Other series of mutants were created by transforming these strains with a particular plasmid to over-express the *OGG1* gene. In chapter 3 the non specific spontaneous mutation frequencies of these mutants were

investigated using a forward mutation assay. In Chapter 4, the frequency of GC to TA mutation was examined in the same mutants using a specific GC to TA reporter system.

Lastly, in chapter 5, a method described by Meniel and Waters, (1999) was used to detect spontaneous and photosensitizer-induced oxidative base damage at the level of nucleotide in the *MFA2* gene. Two photosensitisers, Methylene blue and [R]-1(10-Chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol or RO 19-8022 (RO), which both induce oxidative base damage at the presence of light, were used to induce *in vitro* and *in vivo* oxidative base damage respectively.

Chapter 2

Construction of the mutant strains

2.1 Introduction

Reactive oxygen species are constantly produced in the cells during normal aerobic metabolism or as a result of oxidative stress. ROS can induce mutation via various kinds of DNA damages, as well as damaging other cellular macromolecules. The outcome of such damages can vary from undetectable to lethal. A number of highly conserved repair mechanisms are operating in living organisms to prevent the deleterious consequences of these damages (Friedberg *et al.,* 1995).

A major class of oxidative DNA damages are purine base modifications. Considerable evidence suggests that this kind of oxidative base damage can have biological consequences linked to mutagenesis, carcinogenesis and ageing (Demple and Harrison, 1994). One of the best-studied lesions in this class is 8-oxoguanine (8oxoG). If this lesion remains unrepaired, it can give rise to GC → TA mutations during replication due to its mispairing with adenine instead of cytosine (Shibutani *et al.*, 1991).

In *E.coli* two DNA glycosylases namely Fpg and MutY are responsible for preventing mutations caused by 8-oxoG. The *mut Y* gene product excises adenine in an 8-oxoG:A mispair (Tsai-Wu *et al.*, 1992). On the other hand, the Fpg enzyme operates directly by excising the 8-oxoG lesion itself. In addition to 8-oxoG, several other

forms of DNA damages such as AP sites, 5' terminal deoxyribose phosphate, and imidazol ring opened (FAPY) forms of adenine and guanine can be removed by the Fpg enzyme (Boiteux *et al.*, 1992; Tchou *et al.*, 1994). In support of the critical role of Fpg in removing 8-oxoG, *mut M* strains of *E.coli* defective in Fpg activity, show a five fold increase in the frequency of spontaneous GC to TA transversion mutation (Michaels *et al.*, 1991). They also exhibit a six times higher steady state level of 8-oxoG relative to wild type cells (Besscho *et al.*, 1992).

Enzymes with similar activities have been identified in yeast and mammalian cells (de Oliveria *et al.*, 1994; Bessho *et al.*, 1993). The *OGG1* gene in *S.cerevisiae* have been identified and cloned (Van der Kemp *et al.*, 1996). *OGG1* gene product is a DNA glycosylase/Ap-lyase that can excise 8-OxoG, and to a lesser extent Fapy. A Fapy DNA glycosylase has been identified in *S.cerevisiae* which has a similar molecular weight to *Ogg1* but it exhibits a much higher affinity for Fapy compared to 8-oxoG (de Oliveira *et al.*, 1994). These results suggest that unlike *E.coli*, *S.cerevisiae* possesses two different enzymes to remove 8-oxoG and Fapy sites from DNA (Van der Kemp *et al.*, 1996). This scenario is supported by the cloning of the *NTG1* gene in yeast, which releases FAPY but not 8-oxoG from DNA (Eide *et al.*, 1996).

The above BER enzymes and their biological roles have been discussed in detail in the general introduction chapter (Sections 1.3.2.2 and 1.3.2.3).

Other lines of evidence suggests that NER as well as BER, may have a role in removing oxidative damages from DNA. As discussed in the general introduction chapter (1.4), this possibility has only recently come under focus and is supported by evidence from *E.coli* and Human cells. Experiments with *S.cerevisiae* also suggest

that the broad substrate specificity of NER extends to oxidative base lesions. These data will be discussed in detail in the introduction section of the future chapters.

2.2 The present study

The main aim of the experiments presented in this thesis was to further investigate the contribution of NER and BER to the repair of oxidative base damages in *S.cerevisiae*, by studying the role of these mechanisms in controlling spontaneous and induced mutations in the cells and at the level of nucleotide. In order to perform these experiments, the first step was to construct the reporter strains so that they harbored deficiencies in NER, BER or both. Base excision repair deficient strain were previously created by disruption of the *OGG1* gene via transformation of the yeast cells with an integrating plasmid containing partially homologues sequences to that of *OGG1*. This transformation gave rise to a partial deletion of the coding sequence of the *OGG1* gene by insertion of marker genes; yielding *ogg1::TRP1* and *ogg1:: URA3* mutant strains (Thomas *et al.*, 1997). The same approach was used in the experiments presented in this chapter to create NER deficient strains

To inactivate NER, the *RAD14* gene was disrupted by transforming the yeast cells with an integrating plasmid, which knocks out the *RAD14* gene and replaces it with a *LEU2* marker via a homologous recombination. The *RAD14* gene is indispensable for the recognition of DNA damage prior to the incision step of nucleotide excision repair. Therefore, the *RAD14* deletion mutants are totally defective in NER. This confers an extreme $UV_{245\eta m}$ sensitivity on the *rad14* mutants (Bankman *et al.*, 1992). To create strains with different repair activities, the *RAD14* gene was disrupted in

wild type and *ogg1* strains. In order to confirm the disruption of the *RAD14* gene, a UV sensitivity test was carried out for each strain constructed. To provide additional tools to assess the extent of the contribution of these two mechanisms, another set of mutants were created by transforming yeast strains with a high copy number plasmid so as to over-express the *OGG1* gene.

2.3 Materials and methods

2.3.1. *S.cerevisiae* and *E.coli* strains

***S.cerevisiae* strains**	**Characteristics**	**Source**
FF18733	*MATa,his7-2, leu2-3, trp1-289, ura3-52, lys1-1, 112*	F.Fabre
CD138	As FF18733 except *ogg1::TRP1*	Thomas *et al.*, 1997
YMH4	*MATa, cyc1-706, cyc7-67, ura3-52, leu2-3, 112*	M.Hampsey
YOG4	As YMH4 except *ogg1::URA3*	Thomas *et al.*, 1997

Table 2.1 Description of the *S.cerevisiae* strains manipulated in this study.

***E.coli* strains**	**Characteristics**	**Source**
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (r_k-, m_k+), *rel*A1, *sup*E44, Δ(lac-*pro*AB), [F' *tra*D36, *pro*AB, $laq^qZ\Delta M15$].	Promega

Table 2.2 Description of the *E.coli* strain used in this study.

2.3.2 plasmid pBM190

The integrating plasmid pBM190 for disruption of the *RAD14* gene, was kindly provided by L. Prakash and it's construction was previously described (Banckman *et al.*, 1992). In summary, to construct this plasmid, a 2.25-kb *Bg1II-Xbal* fragment containing the entire *RAD14* ORF together with the 5' and 3' flanking regions was cloned into *Bam*HI-*Xba*l digested pUC19 to create plasmid pBM18. Subsequently, all but the first 40nt of the *RAD14* ORF were replaced by a 2-kb *Hin*dIII-*Sma*I fragment containing the yeast *LEU2* gene, generating the plasmid pBM190. This was used in here for the disruption of the *RAD14* gene (Fig. 2.1; Fig. 2.2). Transformation of the *RAD14* $^{+}$ stains to *LEU* $^{+}$, by pBM190 generates a genomic *Rad14::LEU2* strain (Bankman *et al.*, 1992).

Figure 2.1. Plasmid **pBM188**, made from cloning the entire *RAD14* gene into BamHI-Xbal digested pUC19. The black bar represents the *RAD14* ORF, hatched bars represent the 5' and 3' flanking regions of the *RAD14* gene, and thin lines represent pUC19 sequence. Symbols for restriction enzyme sites: B, *Bam*H1;Bg, *Bg1*II; SP, *Sph*1; X, *Xba*l; H, *Hin*dIII; R, *Eco*RV; Sm, *Sma*I; N, *Nco*l. *Adapted from Bankmann et al.,(1992).*

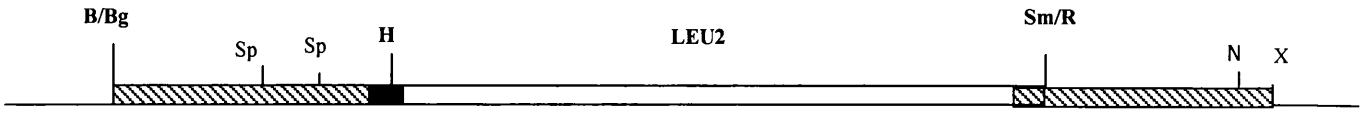


Figure 2.2. Plasmid **pBM190** made from replacement of the all but the first 40 nt of the *RAD14* ORF by a fragment containing *LEU2*. The white bar represents the *LEU2* ORF, the hatched bars represent the 3' and 5'flanking regions of the *RAD14* gene and thin lines represent the pUC19 sequences. Symbols for restriction enzyme sites are as described in Fig.2.1. *Adapted from Bankmann et al., (1992).*

2.3.3 Plasmid pYSB10

The high copy number, non-integrating, recombinant plasmid pYSB10 carries the yeast *OGG1* gene together with the *URA3* selectable marker. It is derived from the plasmid pFL44L, where the transcription of *OGG1* is under the control of the *lacZ* promoter (Van der Kemp *et al.*, 1996).

2.3.4 Storage of the yeast strains

1ml stocks of exponential phase cells were frozen down in Yeast complete or minimal medium (YPD or YNBD respectively) containing 30% glycerol and kept at –70°C. Strains in use were kept on slopes of YPD or minimal medium at 4°C. To avoid contamination, all manipulations were performed in a standard microbiological hood applying aseptic techniques.

2.3.5 Growth conditions of yeast strains

The yeast strains were grown, either in YPD complete medium or, when necessary in YNBD minimal medium with constant agitation at 28°C (Sambrook *et al.*, 1989). Based on the auxotrophic requirements of the yeast strains, adenine (40μg/ml), histidine (100 μg/ml), leucine (100μg/ml), lysine(40μg/ml), uracil(20μg/ml) or tryptophan (20μg/ml) were added to the minimal medium.

2.3.6 Bacterial strains and growth conditions

The bacterial strain JM109 used in this study, is described in table 2.2. The bacterial cells were grown in LB broth at 37°C with vigorous agitation. Solid media contained,

in addition to LB broth, 1.5% agar and was supplemented with 100 μg of ampicillin/ml.

2.3.7 Transformation of *E.coli*

In order to maintain and "bulk up" the plasmid DNA required to transform yeast, the plasmid DNA was introduced into the JM109 *E.coli* strain (Promega). Following the manufacturer's protocol, 17 X 100 mm polypropylene culture tubes were pre-chilled on ice, one per transformation. The frozen, competent cells were then removed from –70°C and placed on ice for 5 minutes or until they had just thawed. The cells were gently mixed by flicking the tubes, and 100 μl of the thawed competent cells were added to each of the pre-chilled culture tubes. 25ng of cloned DNA was then added per 100 μl of competent cells

Tubes were immediately returned to ice for 10 minutes. The cells were heat shocked for 45-50 seconds in a water bath at 42°C with no shaking. The tubes were then placed immediately on ice for 2 minutes. 900 μl of cold LB medium was added to each transforming reaction and the mixture was incubated for 60 minutes at 37°C with shaking. 100 μl of undiluted, 1:10 and 1:100 dilutions of each transforming reaction was spread on to LB plates containing 100μg/ml ampicilin. The plates were incubated at 37°C for 12-14 hours until the transformed colonies appeared.

2.3.8 Plasmid isolation

In order to isolate plasmid DNA from bacterial cells, the QIAprep plasmid kit manufactured by QIAGEN was used. According to the standard protocol supplied

by the manufacturer, 100 ml LB medium was inoculated with transformed bacteria isolated on the LB/ampicilin plates and incubated at 37°C overnight. 5 ml of this culture was spanned and bacterial cells were re-suspended in 250 µl of buffer P1 (containing RNaseA) and were transferred to a microfuge tube. To lyse the bacterial cells under alkaline conditions, 250 µl of buffer P2 (NaOH/SDS) was added and the tube was gently inverted to mix. SDS contained in the buffer P2 solublizes the phospoholipid and protein components of the cell membrane, leading to lysis and release of the cell contents, while the alkaline condition denatures the chromosomal and plasmid DNAs as well as proteins. To provide high salt concentration, 350 µl of buffer N3 was added and the tube was gently inverted 4-6 times, followed by centrifugation for 10 minutes. The high salt concentration causes the denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate while the smaller plasmid DNA renatures and stays in solution. To trap the plasmid DNA, a QIA prep spin column was placed in a 2 µl collection tube and the supernatant from the previous centrifugation was added into a QIA prep column by decanting. The contents were centrifuged for 30-60 sec and the flow through was discarded. In order to remove the residual trace nuclease activity, the QIA prep spin columns were washed by adding 0.5 ml of buffer PB and centrifuged for 30 –60 seconds. The QIAprep spin columns were washed again by adding 0.75 ml of buffer PE and centrifuged for 30-60 seconds. The flow through was discarded and the columns were centrifuged for an additional 1 min to remove residual wash buffer (as the residual ethanol from buffer PE may inhibit subsequent enzymatic reactions). The QIA prep columns were placed in clean 1.5 µl microfuge tubes and plasmid DNA was eluted by adding 50 µl of 10

mM Tris-HCl, pH 8.5 or H_2O to the centre of each QIA prep column which was allowed to stand for 1 min and then centrifuged for 1 minute. The plasmid was then visualised by gel electrophoresis as described next (all the buffers used, were contained in the QIA prep plasmid kit).

2.3.9 Non-denaturing gel electrophoresis

To visualise the plasmid DNA, samples were loaded onto a non-denaturing 1% TAE agarose gel and electrophoresed under 1X TAE running buffer. Gels contained 1 μg per ml Ethidium Bromide (Et Br) to allow visualisation of DNA in the gel using a UV transilluminator. To 2 μl of each DNA preparation, 10 μl of H_2O and 2μl of a non-denaturing loading buffer were added. The non-denaturing loading buffer contained 2.5% Ficol, which increases the density of the solution and aids loading of the DNA samples into the wells of the gel. The dye Bromocresol green was also added to the non denaturing loading buffer; under electrophoresis this migrates at the position of a 200 bp DNA fragment. Electrophoresis at 45 V was carried out for 30-40 min using a Biorad powerpack 300, after which the gel was visualised by UV transillumination (Fig. 2.3)

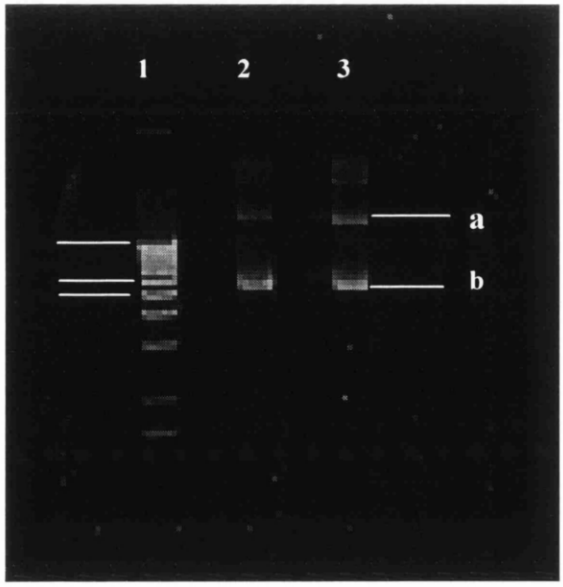


Figure 2.3 An electrophoresis gel photograph of the plasmid DNA. Lane 1, is the 1kb size ladder, lanes 2 and 3, are plasmid pBM190 samples where the two bands in each lane, represent the open circular (a) and super-coiled (b) conformation of the plasmid DNA. (As the plasmid DNA is uncut, the actual size of the plasmid can not be deduced using the size marker).

2.3.10 Disruption of the *RAD14* gene -Transformation of yeast

Disruption of the *RAD14* was carried out by transforming the wild type or the *ogg1* mutant strains with plasmid pBM190, which carries the *LEU2* selectable marker. Transformation was performed using a technique described by Gietz *et al.*, (1992). This method uses Lithium acetate and PEG to induce DNA uptake by making changes in the cell wall that acts as barrier to transformation. The cell wall of cells treated with LiAc (and other agents that induce genetic transformation in yeast), becomes porous and leaky for nucleic acids. For example, it has been observed that RNA can be found in the supernatant of the cells treated with LiAc and SDS but not in the supernatant of the cells treated with SDS alone (Brzobohaty and Kovac 1986). Therefore, in the process of transformation, LiAc probably provide pores through which the vector DNA can enter the cells. On the other hand PEG, being a very viscous solution, enhances transformation by concentrating the carrier and plasmid DNA on to the cell surface (R.D Gietz, unpublished results). To increase the transformation efficiency in this method, denatured salmon sperm DNA is used as carrier, as it has been suggested that single stranded nucleic acids may carry the vector DNA along as they enter the cells. It is also possible that DNA uptake is an active process that can be induced by single stranded DNA (Schiestl and Gietz, 1989). In addition, a heat shock of 42°C is advised in this protocol to increase the efficiency of transformation. The process of transformation described in Gietz *et al.*, (1992) was performed as follows:

10 ml of YPD was inoculated with yeast from an overnight culture so as the culture, when incubated overnight at 28^0C, would reach a density of 10^7 cells/ml. Cells from this culture were used to inoculate a fresh 10 ml YPD medium at a density of 2 x 10^6 cells/ml. The cells were incubated for another 4-5 hours. Re-growth is necessary for maintenance of a plasmid in the strain to be transformed. The culture was centrifuged at 4000 rpm for 5 minutes at room temperature, the cells were resuspended in 10 ml of distilled sterile water and transferred to 15 ml tubes. Cells were collected again by centrifuging as above, resuspended in 1ml of H_2O and transferred to a 1.5 ml Eppendorf tube. Cells were washed twice with 1 ml filter sterile 1 x TE, LiAc (made fresh from 10 X filter sterile stock; 10 X TE; 10 X LiAC). The cells were then resuspended in 100 µl of this solution. 50 µl of yeast suspension, 50 µg of denatured (20 minutes @ 100°C) Salmon sperm carrier DNA and 1.2 µg of transforming DNA were added to each microfuge tube containing 300 µl of freshly made filter sterile 50% PEG solution containing 10%LiAc and 10%TE. The content was mixed well by flicking the tube and the mixture was incubated at 28°C for 30 minutes with agitation. The samples were heat shocked for 15 minutes at 42°C in a preheated heating block. The tubes were centrifuged in a microfuge for 2 minutes and the cells were resuspended in 1 ml of YPD following incubation for 1 hour at 28°C with frequent agitation. The tubes were centrifuged as above and washed twice with 1ml sterile H_2O. Tubes were centrifuged again and the cells were re-suspended in 1ml of 1X TE. 200 µl of each sample was added to selection plates (minus leucine plates) and these were incubated at 28°C, until transformant *LEU*$^+$ colonies appeared.

2.3.11 UV sensitivity test

Disruption of the *RAD14* gene makes the cells highly sensitive to UV light and provides a selectable phenotype, which can be used to confirm that the *RAD14* gene is disrupted in the *LEU2*$^+$ transformants. To test UV sensitivity, cells were grown in YNBD medium at 28°C with constant agitation to reach a density of $1x10^7$cells/ml. The cells were collected by centrifugation, washed and re-suspended in ice cold PBS to a density of $2x10^7$ cells/ml. Cells were then exposed to increasing lengths of time to UV 254 ηm at a dose rate of 3 J/m^2/s using a Philips UV 30W lamp. UV-treated and control mock treated cell suspensions were diluted and a 0.1 ml of the dilutions was spread on YNBD plates from which colonies were scored after incubation for 3 days at 28°C.

2.3.12 Over-expression of the *OGG1* gene

Each strain was transformed with a high copy number plasmid pYSB10, over-expressing the *OGG1* gene. Transformation of *S.cerevisiae* was performed as described earlier. Plasmid pYSB10 carries the *URA3* selectable marker and therefore the transformants were selected on YNB minimal medium on the basis of uracil auxotrophy. As pYSB10 is a non-integrating plasmid and it could be lost from the transformants during cellular replication, it's maintenance was ensured by maintaining cells on minimal media.

2.4 Results

2.4.1 Disruption of the *RAD14* gene – Construction of *rad14* deletion mutants

The following strains were constructed by disrupting the *RAD14* gene via transformation of the cells with plasmid pBM190, which carries the $LEU2^+$ selectable marker. The transformants were initially isolated on the minus leucine plates. The disruption of the *RAD14* gene was then confirmed by undertaking the UV sensitivity test for each transformant. The UV survivals of wild type, *ogg1*, *rad14* and *ogg1rad14* strains are presented graphically in figures 2.4 and 2.5. The *ogg1* mutant was not unusually sensitive to $UV_{254\eta m}$ compared to wild type cells. Disruption of *RAD14* however resulted in extreme sensitivity to $UV_{254\eta m}$, which is consistent with previous reports for these mutants (Bankmann *et al.*, 1992). The raw data are contained in Appendix II.

Strain	**Genotype**
ADS100	**As FF18733 except *rad14::LEU2***
ADS110	**As FF18733 except *ogg1::TRP; rad14::LEU2***
ADS304	**As YMH4 except *rad14:: LEU2***
ADS314	**As YMH4 except *ogg1::URA3* and *rad14::LEU2***

Table 2.3 The *S.cerevisiae* strains constructed by disruption of the *RAD14* gene.

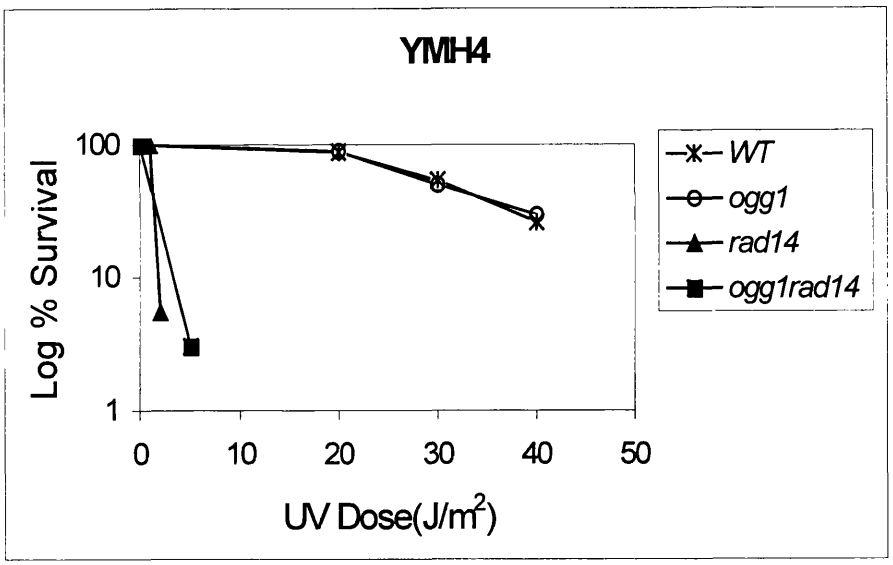


Figure 2.4 UV survival curves for wild type *RAD* (YMH4), *ogg1*(YOG4), *rad14* (ADS304) and *ogg1rad14* (ADS314) cells

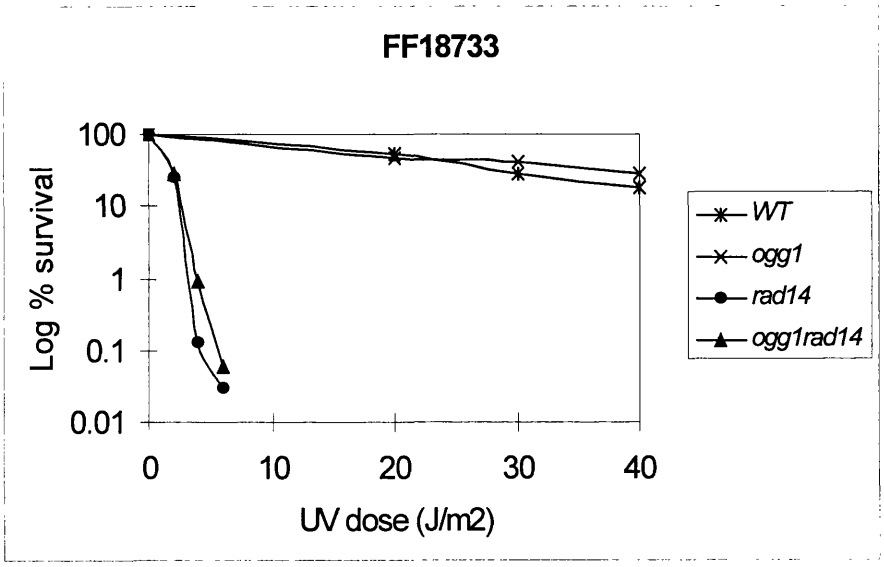


Figure 2.5 UV survival curves for the wild type *RAD* (FF18733), *ogg1* (CD138), *rad14* (ADS100) and *ogg1rad14* (ADS110) strains.

2.4.2 Construction of the *OGG1* over-expressing mutants

The following strains (Table 2.4) were constructed by transformation of yeast by the plasmid pYSB10 carrying the *URA3* selectable marker. The transformants were selected on minus uracil YNBD plates.

Strain	**Genotype**
FF18733 + pYSB10	As FF18733 except that it is *URA3*$^+$
CD138 + pYSB10	As CD138 except that it is *URA3*$^+$
ADS100 + pYSB10	As ADS100 except that it is *URA3*$^+$
ADS110 + pYSB10	As ADS110 except that it is *URA3*$^+$

Table 2.4 *S.cerevisiae* strains transformed by the plasmid pYSB10, which over-expresses the *OGG1* gene.

2.5 Discussion

The *RAD14* gene was disrupted in wild type and *OGG1* deficient *S.cerevisiae* cells to provide strains defective only in NER, and strains defective in both NER and BER, respectively. In addition the wild type, *ogg1,* and the constructed *rad14* and *rad14ogg1* mutants were transformed by plasmid pYSB10 carrying the *URA3* selectable marker that over-expresses the *OGG1* gene under the control of *lacZ* promoter.

These modifications were performed on two different *S.cerevisiae* reporter strains; FF18733 and Hampsey (1991) strains that enable us to detect the effects of these manipulations on the frequency of nonspecific spontaneous mutations (in chapter 3) and spontaneous GC to TA reversion mutations (in chapter 4) respectively.

Thus, the strains constructed during the experiments presented in this chapter, provided the necessary tools to investigate the contributions of NER and BER to the repair of oxidative base damage in *S.cerevisiae* in the experiments described in the next 3 chapters.

Chapter 3

Spontaneous forward mutation to canavanine resistance in *RAD* and *rad14* strains

3.1 Introduction

The role of the *OGG1* gene product of *S.cerevisiae* in the excision repair of oxidative base damage has now been well documented. Using a biochemical approach (Casting *et al.,* 1993; de Oliveirra *et al.*, 1994) it has been shown that the majority if not all, of the enzymatic activity cleaving the 8-oxoGC duplex in *S.cerevisiae* cell free extracts is associated with the Ogg1 protein. Investigating the biological role of the Ogg1 protein demonstrated that *S.cerevisiae* mutants defective in *OGG1* have significantly higher mutation frequencies to Can^R and Lys^+ and that the *ogg1* mutant has a specific GC to TA mutator phenotype (Thomas *et al.*, 1997). These were discussed in detail in general introduction chapter (Section 1.3.2.3).

Northern blot analysis of the total RNA extracted from a yeast wild type strain, has shown that the *OGG1* gene is expressed in very low amounts in wild type cells in normal growth conditions. It was also demonstrated that the over-expression of the *OGG1* gene, using the multy-copy plasmid pYSB10, increased the 8-oxoG cleaving activity in wild type cells up to ten fold. Moreover, these studies have shown that the

mutator phenotype of the *ogg1* mutants can be completely suppressed by the over-expression of the *OGG1* gene using this plasmid. These data strongly confirm the essential role of the BER enzyme Ogg1, in preventing spontaneous mutations resulting from oxidative base damage in *S.cerevisiae* (Thomas *et al.*, 1997).

On the other hand there is certain evidence to suggest that, in addition to BER, NER also has a role in removing such damages. This evidence is principally from research with *E.coli* and to a lesser extent in humans. These events were discussed in detail in the general introduction chapter (section 1.4). More recently, the contributions of NER and BER to the repair of oxidative base damage have been investigated in *S.cerevisiae.*

It has been previously shown that the photosynthesizer methylene blue with light (MB-light) generates base modifications in DNA that can be incised by *Fpg* and the *UvrABC* excinuclease complex (Muller *et al.*, 1990; Czeczot *et al.*, 1991). To further investigate the role of NER in removing oxidative base damages in *S.cerevisiae*, Scott *et al.,* (1999) applied a similar approach to one undertaken by Czeczot *et al.*, (1991) discussed in the general introduction (1.4); pRS413 DNA treated with MB-light, has been used to transform the wild type, *ogg1*, and *ogg1rad14* mutant strains of *S.cerevisiae.* As a measure of repair, the percentage frequency of the surviving transformants have been calculated by comparing the number of transformants obtained following transformation of the cells with equal amounts of treated and untreated plasmid DNA. These results have indicated no decrease in plasmid survival in the *ogg1* strain compared to the isogenic wild type. In contrast, the transformation frequency has been significantly reduced in both the *rad14* and *ogg1rad14* strains.

Interestingly, the reduction in plasmid survival was similar for both these mutants suggesting that the degree of sensitivity to MB-light is solely affected by NER (Scott *et al.*, 1999).

Considering that the principal lesion induced by MB-light is 8-oxoG (Schneider *et al.*, 1990) with moderate toxicity to the living cells (Wood *et al.*, 1990), it has been argued that if the MB-light induced 8-oxoG is responsible for the reduction in plasmid survival in *rad14* cells, then the product of the *RAD14* gene must certainly have a role in the repair of this type of base damage. Since the plasmid survival of the *ogg1* mutant was not significantly lower than that of the wild type, and no further decrease in the plasmid survival was observed in *rad14ogg1* mutants compared to the single *rad14* mutant, it has been suggested that NER rather than the *Ogg1* base excision repair activity is the major mechanism involved in removing 8-oxoG in *S.cerevisiae.* However, as the spontaneous GC → TA mutator phenotype of the *ogg1* mutants confirm a role for *OGG1* in the repair of 8-oxoG *in vivo*, it is more likely that the toxicity induced by MB-light is caused by DNA damages other than 8-oxoG. In support of this hypothesis, the possible formation of fomamidopyrimidine residues in DNA following MB light treatment has not been excluded (Czeczot *et al.*, 1991). Moreover, it has been shown that the purified Ogg1 protein has greater affinity for 8-oxoG compared to Fapy in double stranded DNA (Van der Kemp *et al.*, 1996). Therefore, the absence of an additional decrease in plasmid survival in the *ogg1rad14* cells compared to the *rad14* strain is possibly due to the *ogg1* having a reduced Fapy DNA glycosylase activity. This does not exclude the possibility of existence of an additional glycosylase which also releases Fapy from DNA, suggested by Van der Kemp *et al.*, (1996). The recent characterization of the *NTG1* gene that encodes for a

Fapy DNA glycosylase in *S.cerevisiae* (Eide *et al.*, 1996) supports this hypothesis. Whatever the case, the results of plasmid survival experiments in *S.cerevisiae,* indicate a role for NER in the removal of some damages induced by MB-light (Scott *et al.*, 1999).

Interestingly, the results of the plasmid survival experiments in *S.cerevisiae* are very different to what has been reported for *E.coli.* This bacterium demonstrates a decrease in transformation efficiency in the *fpg/uvrA* mutants comparing to either single mutant, neither of which shows a reduction in plasmid survival relative to the wild type. This suggests that in *E.coli*, Fpg and NER proteins both have a role in removal of oxidative base lesions *in vitro* and they compete for the same substrates. Therefore, the loss of one activity can be compensated for by the other (Czeczot *et al.*, 1991).
To confirm that the differences in the results of plasmid survival experiment between *E.coli* and yeast were not due to the use of different plasmids, the experiments of Czeczot (1991) were repeated with plasmid pRS413, and the *E.coli* data were consistent with the previous findings of Czeczot. These result indicate that the differences are either due to different substrate specificities of NER and BER repair proteins in these two organisms, or that they reflect a 'division of labor' in yeast depending on where the substrates are located in the genome (Scott *et al.*, 1999).

Other evidence also suggests a role for NER in the removal of oxidative base damages in *S.cerevisiae.* The Endonuclease III of *E.coli* is a BER enzyme with an unusual broad substrate specificity. Pyrimidine base modifications are among the lesions that can be recognised by endonuclease III and it has been shown that these lesions can be repaired by the UvrABC endonucleases in *E.coli* (Lin and Sancar, 1989; Kow *et al.*,

1990). On the other hand, Reed *et al* has shown that a small group of UV induced lesions detected by their sensitivity to Endonuclease III, are repaired by NER in *S.cerevisiae* (Reed *et al.,* 1996a). In the light of these experiments and those of Czeczot *et al.*, (1991) the role of NER in the removal of ionizing radiation induced purine base modifications has been further investigated by studying the repair of X-ray induced lesions detected in the MATa and HMLα loci of *S.cerevisiae* via their sensitivity to the Fpg protein. These results indicate that the repair of ionizing induced lesions is proficient in wild type cells but is absent in the *rad2* and *rad3* NER mutants. However, the *rad2* and *rad3* mutants, although defective in the repair of these X-ray induced lesions, are not unusually sensitive to X-irradiation (Scott *et al.,* 1999). Also, the principle lethal lesions induced by X-irradiations are DNA single and double strand breaks that can not be repaired by NER (Frankenberg-Schwager and Frankenberg, 1990). Thus, the lesions detected in the Scott *et al.*, (1999) experiments do not contribute significantly to cell killing. The contribution of NER to the repair of oxidative base lesions have been supported by some other experiments which will be discussed in future chapters.

3.2 The present study

The aim of the experiments in this chapter is to further investigate the contribution of BER and NER mechanisms to the repair of spontaneous base damages in *S.cerevisiae* by looking at the effect of disruption of the *RAD14* gene on the frequency of spontaneous mutations in yeast cells. In this respect a forward mutation assay has been used to determine the spontaneous mutation frequencies to Can^R in the wild type, *ogg1* and in *rad14* and *ogg1rad14* mutants of *S.cerevisiae.* These were

constructed in chapter 2 by disruption of the *RAD14* gene in the wild type and *ogg1* mutants respectively. Furthermore, in order to determine whether the loss of the NER activity can be compensated by over-expression of *OGG1*, the same assay has been used to look at the effect of the over-expression of the *OGG1* gene on the frequencies of the spontaneous mutations to Can^R in transformants, harboring the high copy number plasmid pYSB10 that over-expresses the *OGG1* gene, (description of construction in chapter 2) . In order to confirm that the differences in the results of the Canavanine assay for these strains were not due to the plasmid pYSB10 not being over-expressed in some of these strains, the total RNA was extracted from these strains and a northern blotting was performed using an *OGG1* probe.

3.3 Materials and methods

3.3.1 *S.cerevisiae* strains used in this study

Strain	Genotype
FF18733	WILD TYPE
CD138	*ogg1::TRP1*
ADS100	*rad14::LEU2*
ADS110	*ogg1::TRP1, rad14::LEU2*
Harboring plasmid pYSB10 (over-expressing the *OGG1* gene)	
FF18733 + pYSB10	As FF18733 except that it is $URA3^{+}$
CD138 + pYSB10	As CD138 except that it is $URA3^{+}$
ADS100 + pYSB10	As ADS100 except that it is $URA3^{+}$
ADS110 + pYSB10	As ADS110 except that it is $URA3^{+}$

Table 3.1 *S.cerevisiae* strains used in chapter 3 experiments.

3.3.2 The canavanine assay

The strains FF18733 and it's derivatives , were used to report on forward mutation to canavanine resistance. Canavanine is a toxic analogue of arginine. The permeability of the cells to canavanine is controlled by the arginine permease protein. Therefore, in cells with an active arginine permease gene, canavanine can enter the cells and kill them. In cultures of *S.cerevisiae*, grown under normal conditions a canavanine resistant mutation can arise as a result of a forward mutation that inactivates the

arginine permease enzyme. The frequency of appearance of canavanine resistant mutants can be calculated and is indicative of the frequency of spontaneous mutation at this locus. The canavanine assay was performed as follows:

Yeast cells were grown in 2 ml YNBD medium at 28°C for 2 days to a density of ~1 X 10^8 cells/ml. Cultures were obtained by incubation at an initial density of 2.5 x10^4 cells/ml. Experiments were repeated 3-4 times for each strain using 6-8 colonies in each assay to ensure representative data. Cell density was measured by plating 0.1ml dilutions on YNBD agar plates, that were scored after incubation for 3 days at 28°C. To quantify the number of canavanine resistant (Can^R) mutants, 0.1-0.4 ml of undiluted culture was plated on YNBD plates containing 60 mg/ml of canavanine sulphate. The number of colonies on the plates were scored after incubation for 3-4 days at 28°C and the frequency of spontaneous mutation was calculated according to the following formula:

$$\text{Mutation frequency} = a/(b \times c \times d).$$

Where: a = average number of colonies on the YNBD + canavanine plate

b= average number of colonies on the YNBD plate.

c= YNBD plate dilution

$$d = \frac{\text{Volume of the undiluted culture spread on the YNBD+canavanine}}{\text{Volume of the diluted culture spread on the YNBD plate.}}$$

3.3.3 RNA extraction

To extract the total RNA from the *S.cerevisiae* cells, a simple and rapid method described by Schmitt *et al.*, (1990) was used as follows:

10 ml cultures were grown in YPD or minimal medium to an OD_{600} of 2.5-5. The cells were harvested by centrifugation and re-suspended in 400 µl of 50 mM Na acetate pH 5.3, 10 mM EDTA (AE buffer). The resuspended cells were transferred to a 1.5 ml microcentrifuge tube and to lyse the cells, 40 µl of 10% SDS was added. The suspension was vortexed and in order to extract the proteins from the solution, an equal volume of phenol, previously equilibrated with AE buffer was added. The mixture was then vortexed and incubated at 65°C in a water bath for 4 minutes. The mixture was then rapidly chilled in a dry ice/Ethanol bath until phenol crystals appeared and was then centrifuged for 2 minutes at a maximum speed in a microcentrifuge to separate the aqueous and phenol phase. The upper, aqueous phase was transferred to a fresh microcentrifuge tube and to further purify the solution from the proteins and cellular debris, it was extracted with phenol chloroform at room temperature for 5 minutes. The extracted aqueous phase was then brought to 0.3M sodium acetate, pH 5.3, by adding 40 µl of 3 M sodium acetate pH 5.3, after which 2.5 volumes of ethanol were added to precipitate the RNA. After washing with 80% ethanol, the pellet was dried and resuspended in 20 µl of sterile water, and stored at – 70°C until used. Throughout the preparation, normal precautions to avoid ribonuclease contamination were taken. (For each strain total RNA was extracted from 100 ml *S.cerevisiae* cultures).

3.3.4 RNA gel electrophoresis

Like DNA, RNA migrates at different rates in relation to its molecular weight. In this process it is necessary to ensure that the RNA does not form secondary structure or

hybridise to another RNA molecule. To ensure that the RNA remains single stranded, the most common way is to react the free amines of the bases with formaldehyde. The resulting Schiff base is not able to form hydrogen bonding between the complementary bases. This will reduce the possibility of the two strands of RNA hybridising or forming secondary structure. Therefore, formaldehyde is contained in the RNA gels. The gel electrophoresis of RNA was performed according to the instruction supplied in the Gene Screen TM protocol (Method of use U.S.patent 4,455,370)

A 150 μg sample of total RNA was dried down, 20 μl of loading buffer was added to each dry sample and the sample was denatured for 2 minutes at 95°C and then cooled on ice for 2 minutes. Using 1X MOPS as a running buffer, samples were loaded and the gel was run until bromophenol blue migrated to three fourths of the gel. The gel electrophoresis was performed in a fume hood to avoid toxic fumes from the formaldehyde contained in the gel. The gel was stained with Ethidium bromide solution and visualized under a UV lamp. (Fig. 3.1)

3.3.5 Designing DNA probes

The sequence of the *OGG1* was downloaded from the seqnet data base and suitable primer sites were identified using the computer software Oligo (Version 3.4 MedProbe. Norway). The software uses mathematical algorithms to allow optimal primers to be designed which form specific and stable primer –template duplexes with the sequence of interest, with minimal self-complementarity and dimer formation. Having selected suitable primer sites, the two primers were ordered from Oswell:

Primer 1 : CCAACCAGCGTATGATCCCCA

Primer 2 : CTGGAATTTGTTGCTGTCGGC

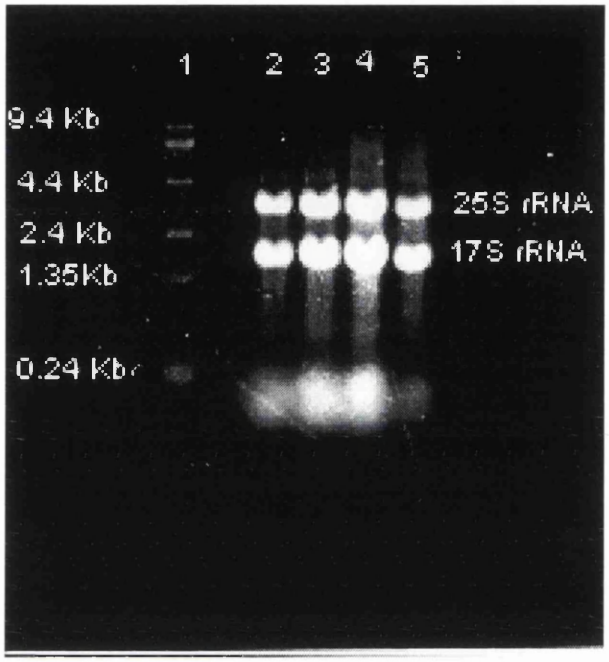


Figure 3.1 Panel 1 shows the 0.24-9.5kb RNA ladder (3μg/lane), Panels 2-5 show total RNA (3 μg/lane) derived from different strains, separated by agarose gel electrophoresis in the presence of formaldehyde, and stained with ethidium bromide. The two intense bands in lanes 2-5 represent 25S rRNA and 17S rRNA.

3.3.6 Preparing the *OGG1* probe by PCR

The probe for *OGG1* was prepared using standard PCR-based amplification of genomic DNA. 30 rounds of PCR with different annealing temperature and different concentrations of $MgCl_2$ was performed on yeast chromosomal DNA. The products from the PCR reaction were loaded on to a 6% polyacrylamide gel and visualized following silver staining. PCR reactions were optimized until a single PCR product was obtained of the correct size relative to the size markers. Fig.3.2. (An annealing temperature of 57°C, a chromosomal DNA concentration of 0.4 μg/ml and $MgCl_2$ concentration of 6μl per 100 μl of the reaction mixture, gave a single product of the correct size corresponding to the *OGG1* probe).

3.3.7 Northern transfer of RNA to nitrocellulose filter

After gel electrophoresis, the gel was soaked in 5 volumes of distilled water for about 5 minutes to remove the formaldehyde from the gel. The nitrocellulose hybridization membrane (Gene screen plus. NEN-Dupont) was cut to the exact size of the gel. The gel was first soaked in distilled water for a few seconds until fully hydrated and was then soaked in 10 X SSPE for 15 minutes. The RNA was transferred from the agarose gel to the hybridization membrane by using a Posiblot 30-30 pressure blotter (Stratagene Ltd. UK.). Transfer was carried out for 4 hours at 80 mm Hg. The membrane was then carefully removed and left on a piece of filter paper to dry overnight. The complete transfer of RNA to the nitrocellulose was confirmed by removing the gel from the Posiblot apparatus for staining with EtBr (Ethidium Bromide).

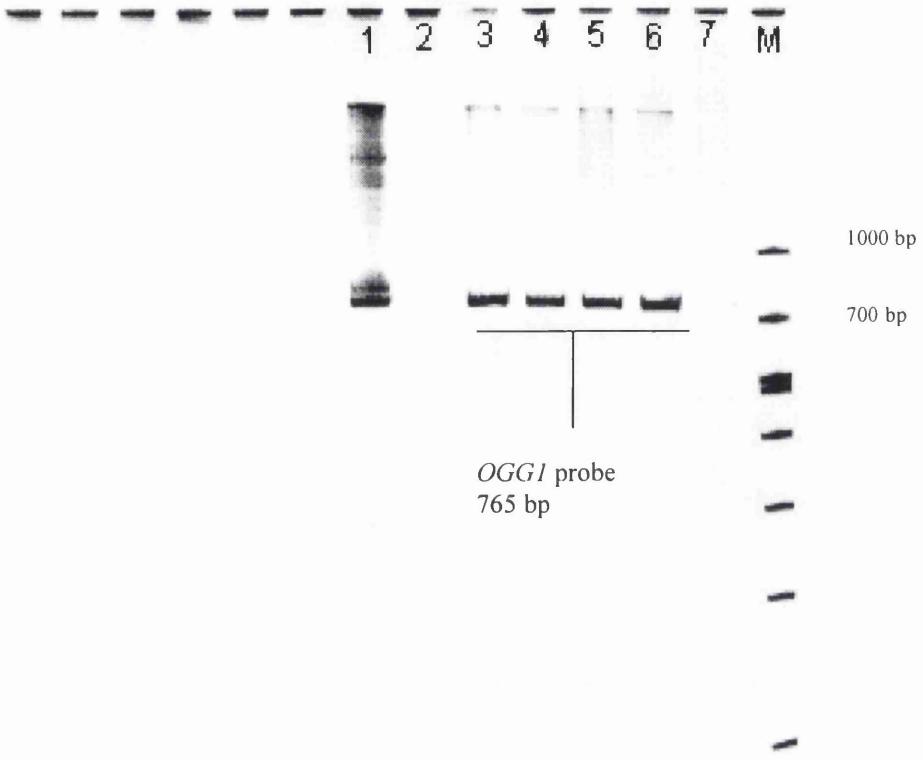


Figure 3.2 Polyacrylamide gel electrophoresis of the PCR amplified probe for the *OGG1* gene. Lanes 1-7 represent different PCR products, obtained in different PCR conditions. The last lane marked M, represents the size marker ladder (Gel marker ^{TM} 1000-50 bp). The PCR products 3-6 representing one intense band of 765 bp, were stored for use as the *OGG1* probe.

3.3.8 Radioactive Random labelling the *OGG1* probe

The probe DNA was labelled using a random primer labelling kit (Prime-It ® II. Stratagne Ltd. UK). The detail of the procedure were as follows:

23 µl of the template DNA (1ηg/µl) was added to 10 µl of random oligonucleotide primers and boiled in an Eppendorf tube for 5 min. This step denatures the double stranded template DNA and as the reaction cools to room temperature, the random primers anneal at multiple sites. 10 µl of 5X primer buffer containing dATP, dGTP, and dTTP (0.1mM of each) was then added to the reaction, followed by addition of 5µl of [α-$_{32}$P] dCTP (3000Ci/mmole) [Amersham International plc.UK]. At the next step, 1 µl of Exo(-) Klenow enzyme (5 units/µl) was added and the reaction was mixed gently by pipetting up and down. Finally, the reaction mixture was incubated at 37°C in a preheated heating block for 10 min. The Exo(-) Klenow enzyme is a 3' exonuclease-deficient mutant of the Klenow subunit of DNA polymerase I. This enzyme can recognize the primer template complexes as substrate and synthesise new DNA by incorporating nucleotide monophosphates at the free 3' OH group provided by the primer. As much as 59% of the total labeled nucleotide can be incorporated in to a 3kb template. Following incubation, the reaction was terminated by addition of 2 µl stop solution (0.5M EDTA. PH8.0). Before the hybridization, the unincorporated nucleotides were removed from the reaction by using a Nuc Trap purification column [Nuc Trap®probe purification. Stratagene Ltd.UK]. These columns trap the unincorporated nucleotides and oligonucleotides smaller than 17bp in length, while the larger labeled probe DNA is eluted. This process is estimated to be 99% efficient. Following these process, the probe and 100µl of denatured salmon sperm DNA (10mg/ml) contained in another Eppendorf tube were boiled in a water bath for 5 min

to dissociate the double stranded DNA. They were then rapidly chilled on ice for a further 5 minutes to ensure strand re-association was minimal.

3.3.9 Northern hybridization

The membrane was prehybridised in a glass bottle [Hybad Ltd. UK] at 65°C for 2 hours in a Hybad hybridisation oven. After pre-hybridisation, the [^{32}P] labeled probe DNA was added along with 100 µl denatured salmon sperm DNA to the pre-hybridisation mixture. The hybridization was allowed to occur overnight at 65°C.

After overnight hybridisation, the membrane was washed with 100 ml of 2 X SSC at room temperature, with 2X SSC, 1X SDS at 45°C for 15 minutes and finally with 0.1% SSC at room temperature. These washes are done to remove nonspecifically bound probes and reduce background signals. Decreasing the salt concentration at each wash increases the stringency of the wash solutions. The membrane was placed on blotting paper, wrapped in cling film and placed under a phosphor-imager screen which was then scanned with a phosphor-imager scanner (Molecular Dynamics, Sunnyvale, CA, USA). The image was then stored in Imagequant software (Molecular Dynamics, Sunnyvale, CA, USA). The membrane was kept in an X-ray cassette and was used for northern hybridization with a *URA3* probe using the same labeling processes.

All of the experiments involving [^{32}P] were carried out in Radioisotope laboratories at special workstations behind Perspex protective screens [Stratagene Ltd. UK]. To avoid radioactive contamination, before, during and after finishing each experiment, the workstations were monitored using a Geiger counter and special care was taken to

avoid any apparatus or personal contamination. To minimise the radioactive exposure, pippettes fitted with Perspex shields were used and in order to monitor the monthly amount of exposure to radioisotope radiation, finger and body badges were used throughout the experiments. All the radioactive waste was stored in a Perspex container until the isotope had decayed to background levels, before incineration.

3.4 Results

3.4.1 Spontaneous mutation frequencies to Can^R in strains with different repair activities

The contribution of BER to the repair of oxidative base damage is relatively well documented and BER enzymes such as Ogg1, Ntg1 and Ntg2 have been identified. The biological role of the *OGG1* gene has been investigated and the studies revealed that *ogg1* mutants exhibit a mutator phenotype. Significant increase in the frequency of spontaneous mutations to Can^R were reported for the *ogg1* mutant relative to the wild type (Thomas *et al.*, 1997). Meanwhile, as discussed in the introduction, there is a considerable amount of evidence suggesting that BER is not the only mechanism operating on oxidative base lesions, and NER may contribute to the repair of such damage. In order to further study the relative contributions of NER and BER to the repair of spontaneous base damage in *S.cerevisiae*, a series of experiments were performed to determine the effect of the inactivation of NER which was achieved by disruption of the *RAD14* gene.

The frequency of spontaneous mutations to Can^R was determined first in the wild type and *ogg1* strains to provide references. Subsequently, mutation to Can^R was determined in the same wild type and *ogg1* strains in which the *RAD14* gene was disrupted as explained in chapter 2. This created *rad14* and *ogg1rad14* mutants respectively. The following sections will first compare the mutation frequencies to Can^R between each strain and the wild type. Subsequently, the frequency of spontaneous mutation to Can^R will be compared between all 4 strains.

3.4.1.1 The frequency of spontaneous mutation to Can^R in an *ogg1* mutant compared to the wild type

In accord with previous findings, the mutants defective in the *OGG1* gene, displayed significantly higher frequency of mutation to Can^R relative to the wild type (Scott *et al.*, 1999). Here, an average increase of 9 fold was detected in the frequencies of spontaneous mutations to Can^R in the *ogg1* mutants compared to the wild type strain.

Table 3.2 summarizes the frequencies of Can^R mutants in the wild type (FF18733), and it's *OGG1* deficient derivative (CD138) compared in 7 independent experiments. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of experiments 3.1-3.7 demonstrating the same pattern of increase are illustrated graphically as bar charts in Fig. 3.3. The raw data are contained in Appendix III.a (A III.a.1-A III.a7). *The statistical terms and the statistical analysis test used to analyze the data in this chapter and chapter 4 are described in appendix V.*

	Can^R/10^6				
	wild		*ogg1*		
	Median	**(LIQV,UIQV)**	**Median**	**(LIQV,UIQV)**	*ogg1* /wild
Exp. 3.1	**0.21**	(0.17, 0.21)	**1.39**	(1.36, 1.41)	7
Exp. 3.2	**0.59**	(0.48, 0.72)	**3.28**	(3.22, 4.42)	6
Exp. 3.3	**0.36**	(0.31, 0.42)	**3.28**	(2.33, 3.92)	9
Exp. 3.4	**0.17**	(0.07, 0.23)	**3.27**	(2.39, 4.37)	19
Exp. 3.5	**0.19**	(0.18, 0.21)	**1.17**	(0.78, 1.47)	6
Exp. 3.6	**0.24**	(0.23, 0.26)	**1.07**	(0.71, 2.20)	4
Exp. 3.7	**0.23**	(0.23, 0.38)	**2.61**	(2.29, 2.65)	11

Table 3.2 Spontaneous mutation frequency to Can ^{R} in the wild type and *ogg1* mutants of *S.cerevisiae.*

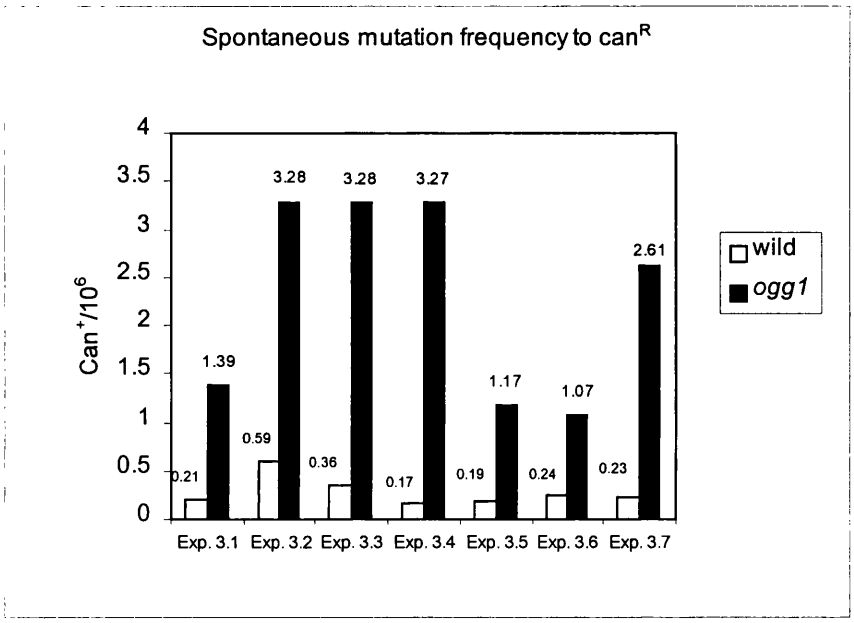


Fig. 3.3 Bar charts comparing the mutation frequencies to Can^R between the wild type and the *ogg1* mutants.

3.4.1.2 Effect of disruption of the *RAD14* gene in wild type on the frequencies of spontaneous mutations to Can^R.

In order to determine the effect of the inactivation of NER, the frequency of spontaneous mutations to Can^R was determined in a single *rad14* strain constructed in the previous chapter. In support of a role for NER in controlling spontaneous mutation in *S.cerevisiae*, this mutant showed a significant increase in the frequency of spontaneous mutation to Can^R (Scott *et al.*, 1999). An average of a 9 fold increase in the frequency of spontaneous mutations to Can^R was observed in the *rad14* mutant strain compared to the wild type. This level of increase in spontaneous mutation is not significantly different from the values obtained for single *ogg1* mutant relative to the wild type (Scott *et al.*, 1999).

Table 3.3 summarizes the frequency of Can^R mutants in wild type (FF18733) and *rad14* single mutant (ADS100) compared in 7 independent experiments. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of experiments 3.1-3.7 demonstrating the same pattern of increase, are illustrated graphically as bar charts in Fig. 3.4. The raw data are contained in Appendix III.a (A III.a.1-A III.a.7).

	Can^R/10^6				
	wild		*rad14*		
	Median	**(LIQV,UIQV)**	**Median**	**(LIQV,UIQV)**	*rad14*/wild
Exp. 3.1	**0.21**	(0.17, 0.21)	**1.52**	(1.26, 2.09)	7
Exp. 3.2	**0.59**	(0.48, 0.72)	**2.73**	(2.34, 3.69)	5
Exp. 3.3	**0.36**	(0.31, 0.42)	**2.04**	(1.95, 2.31)	6
Exp. 3.4	**0.17**	(0.07, 0.23)	**2.24**	(1.88, 3.81)	13
Exp. 3.5	**0.19**	(0.18, 0.21)	**2.48**	(2.41, 2.76)	13
Exp. 3.6	**0.24**	(0.23, 0.26)	**2.34**	(2.15, 2.50)	10
Exp. 3.7	**0.23**	(0.23, 0.38)	**2.24**	(2.20, 2.35)	10

Table 3.3 Spontaneous mutation frequency to Can ^{R} in the wild type and *rad14* mutants of *S.cerevisiae.*

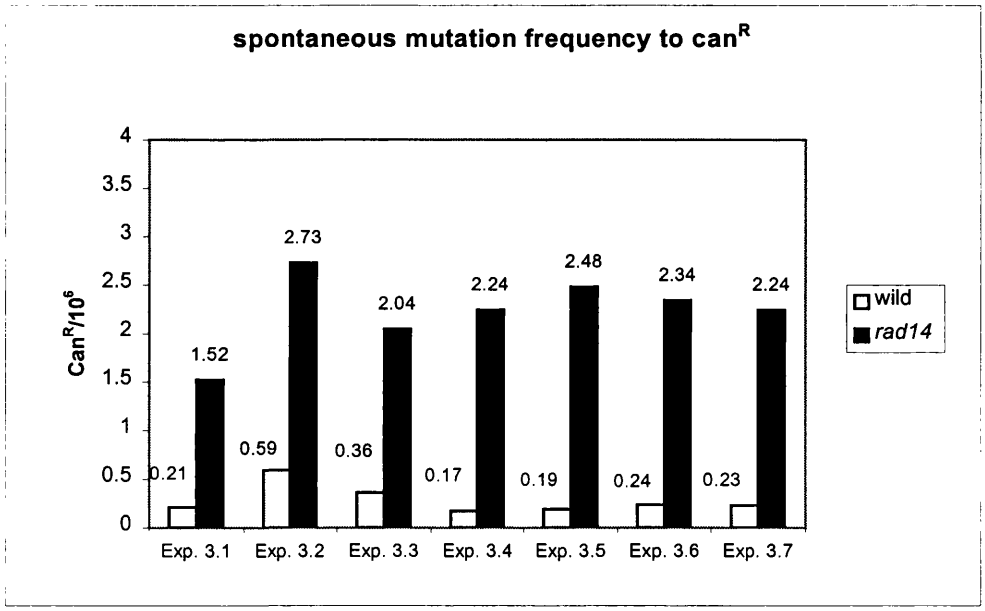


Fig. 3.4 Bar charts comparing the mutation frequencies to Can^R between the wild type and the *rad14* mutants.

3.4.1.3 The frequency of spontaneous mutations to Can^R in a strain defective in both the *RAD14* and *OGG1* genes

In order to further investigate the contribution of NER and BER and to examine whether they are operating on the same lesions, the frequency of spontaneous mutation to Can^R was determined in an *ogg1rad14* double mutant constructed in the previous chapter by disruption of the *RAD14* gene in an *OGG1* deficient background.

The *ogg1rad14* mutant exhibited a significant increase in spontaneous mutations to Can^R relative to the wild type (Scott *et al.,* 1999). An average of a 7 fold increase in the frequency of spontaneous mutation to Can^R was observed in the *ogg1rad14* mutant compared to the wild type. This increase is not significantly different from those obtained for either the *ogg1* or *rad14* single mutants (Scott *et al.*, 1999).

Table 3.4 summarizes the frequencies of Can^R mutants in the wild type (FF1873) and it's NER and BER deficient derivative *ogg1rad14* mutant (ADS110) compared in 7 independent experiments. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of experiments 3.1-3.7 showing the same pattern of increase are illustrated graphically as bar charts in Fig.3.5. The raw data are contained in Appendix III.a (A III.a.1-A III.a.7).

	Can^R/10^6				
	wild		*ogg1rad14*		
	Median	(LIQV,UIQV)	Median	(LIQV,UIQV)	*ogg1rad14* /wild
Exp. 3.1	**0.21**	(0.17, 0.21)	**1.31**	(0.92, 2.24)	6
Exp. 3.2	**0.59**	(0.48, 0.72)	**1.87**	(1.28, 2.40)	3
Exp. 3.3	**0.36**	(0.31, 0.42)	**2.22**	(1.49, 2.40)	6
Exp. 3.4	**0.17**	(0.07, 0.23)	**1.52**	(1.27, 3.39)	9
Exp. 3.5	**0.19**	(0.18, 0.21)	**2.61**	(2.23, 3.28)	14
Exp. 3.6	**0.24**	(0.23, 0.26)	**1.96**	(1.79, 2.13)	8
Exp. 3.7	**0.23**	(0.23, 0.38)	**1.4**	(1.31, 1.65)	6

Table 3.4 Spontaneous mutation frequency to Can ^{R} in the wild type and *ogg1rad14* mutants of *S.cerevisiae.*

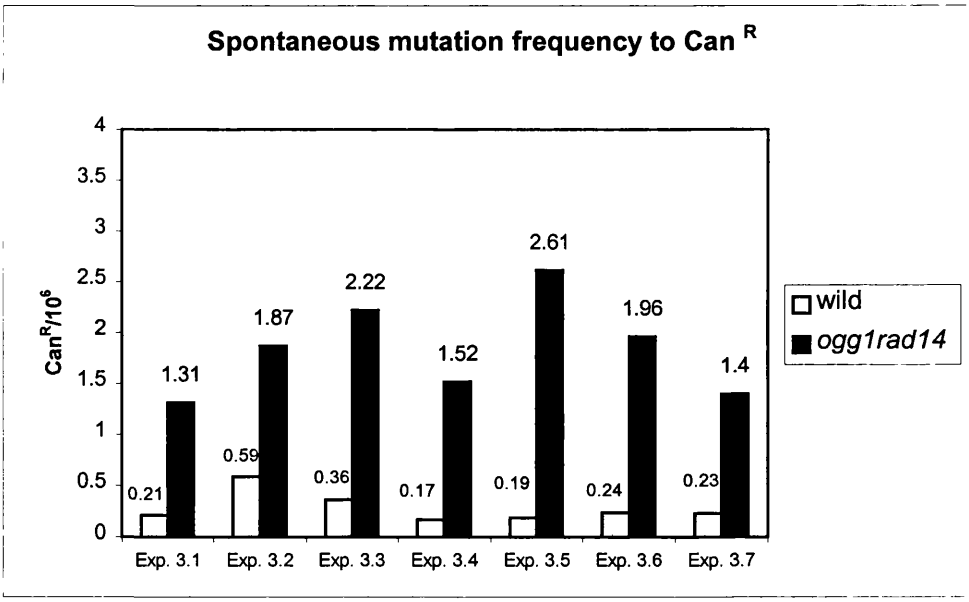


Fig. 3.5 Bar charts comparing the mutation frequencies to Can^R between the wild type and the *rad14* mutants.

In each of experiments 3.1-3.7 all four strains (wild type, *ogg1, rad14, ogg1rad14*) were examined under the same experimental conditions except for the strain specific amino acid supplement to the minimal media. All these experiments demonstrate the same trend: While there is no statistically significant difference in the frequency of spontaneous mutation between the *ogg1, rad14 and ogg1rad14* mutants, they all show a significant increase in that relative to the wild type (Scott *et al.*, 1999).

Tables 3.5 to 3.8 compare the frequencies of Can^R mutants between the wild type (FF1873), *ogg1*(CD138), *rad14*(ADS100) and o*gg1rad14*(ADS110) mutants in 4 of these experiments (Experiments 3.1-3.4). The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. These results are represented graphically as bar charts in Fig. 3.6 to 3.9. The raw data and the statistical analysis for the above experiments are contained in Appendix III.a (A III.a.1-AIII.a.7) and Appendix III.b (A III.b.1-A III.b.7) respectively

Exp. 3.1			
Genotype	**Can^R/10^6**		
	Median	**LIQV**	**UIQV**
wild	0.21	0.17	0.21
ogg1	1.39	1.36	1.41
rad14	1.51	1.26	2.09
ogg1rad14	1.31	0.92	2.24

Table 3.5 The frequency of spontaneous mutation to Can^R compared between the wild type, *ogg1* , *rad14* and *ogg1rad14* strains .

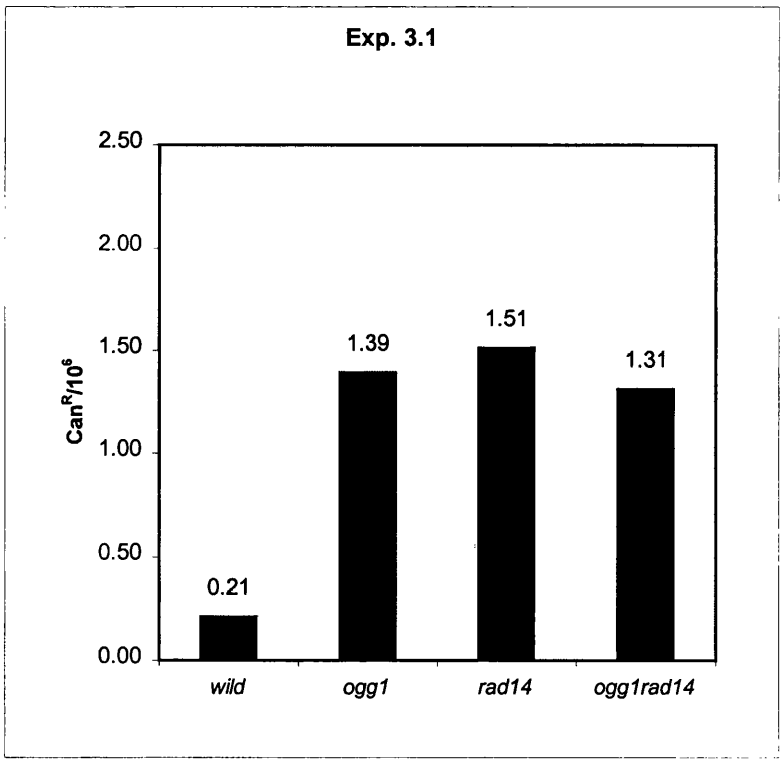


Fig. 3.6 Bar charts comparing the frequency of spontaneous mutations in the wild type, *ogg1, rad14* and *ogg1rad14* strains.

Exp. 3.2			
Genotype	**Can^R/10^6**		
	Median	**LIQV**	**UIQV**
wild	0.59	0.48	0.72
ogg1	3.28	3.22	4.42
rad14	2.73	2.33	3.69
ogg1rad14	1.86	1.28	2.4

Table 3.6 The frequency of spontaneous mutation to Can^R compared between the wild type, *ogg1* , *rad14* and *ogg1rad14* strains .

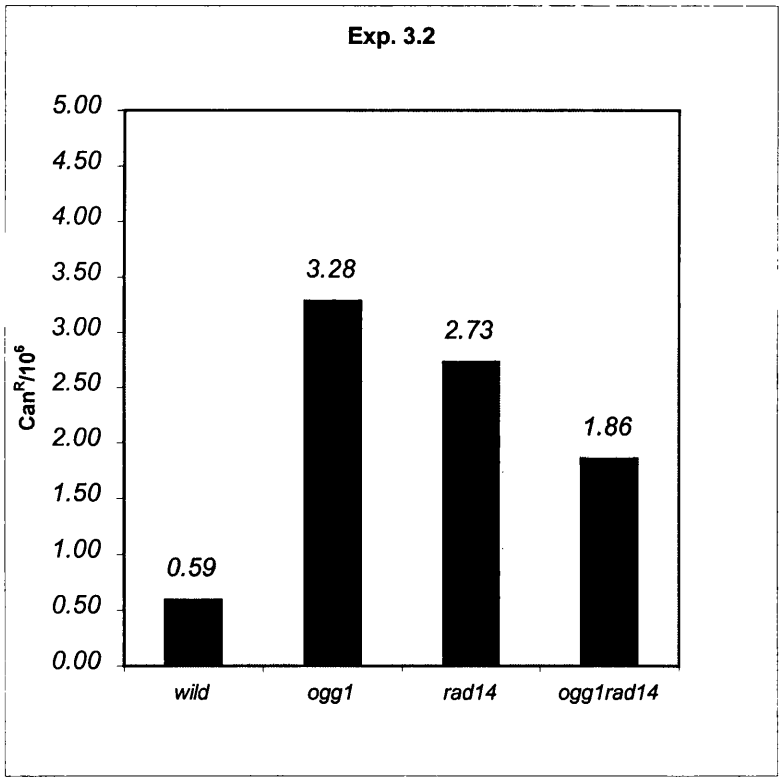


Fig. 3.7 Bar charts comparing the frequency of spontaneous mutations in the wild type, *ogg1, rad14* and *ogg1rad14* strains.

Exp. 3.3			
Genotype	**Can^R/10^6**		
	Median	**LIQV**	**UIQV**
wild	0.36	0.31	0.42
ogg1	3.28	2.33	3.92
rad14	2.04	1.95	2.31
ogg1rad14	2.22	1.49	2.4

Table 3.7 The frequency of spontaneous mutation to Can^R compared between the wild type, *ogg1* , *rad14* and *ogg1rad14* strains .

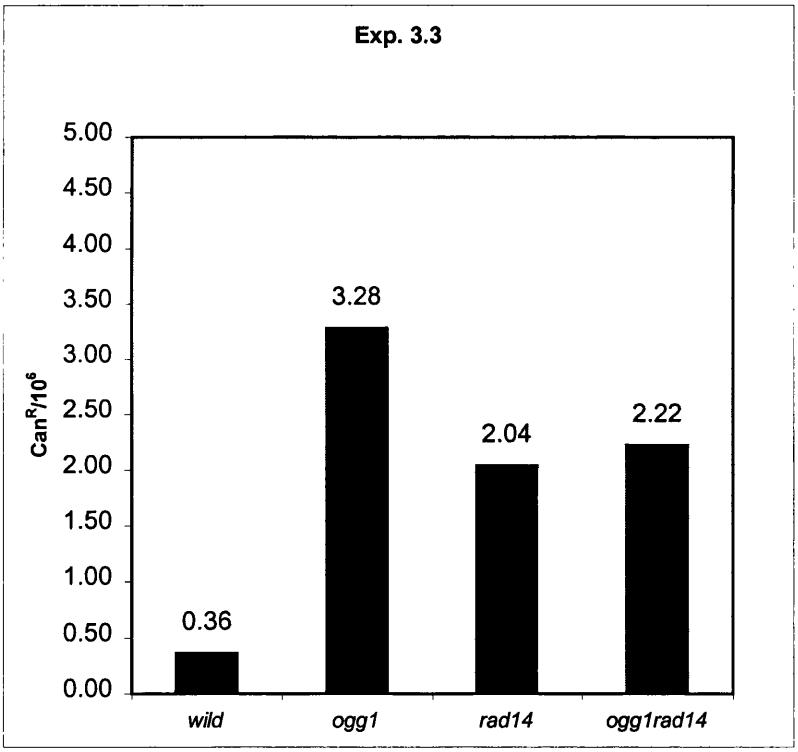


Fig. 3.8 Bar charts comparing the frequency of spontaneous mutations in the wild type, *ogg1, rad14* and *ogg1rad14* strains.

Genotype	Exp. 3.4 Can^R/10^6		
	Median	**LIQV**	**UIQV**
wild	0.17	0.07	0.23
ogg1	3.27	2.38	4.37
rad14	2.24	1.88	3.81
ogg1rad14	1.52	1.27	3.38

Table 3.8 The frequency of spontaneous mutation to Can^R compared between the wild type, *ogg1* , *rad14* and *ogg1rad14* strains .

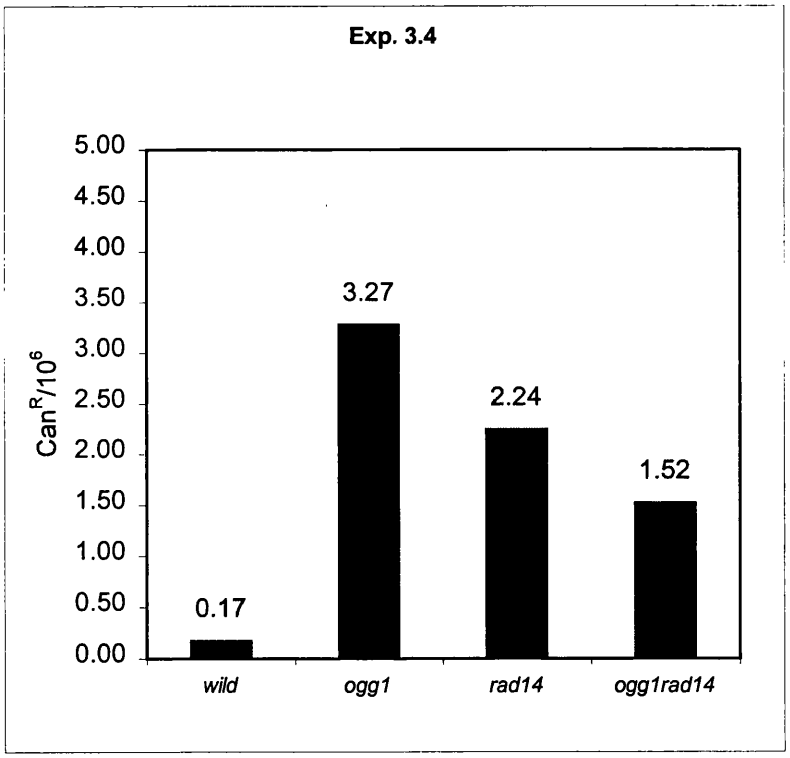


Fig. 3.9 Bar charts comparing the frequency of spontaneous mutations in the wild type, *ogg1, rad14* and *ogg1rad14* strains.

3.4.2 The effect of the over-expression of the *OGG1* gene on the frequency of spontaneous mutations to Can^R

Previously, it has been demonstrated that transformation of the *ogg1* mutant with a high copy number plasmid pYSB10 over-expressing the *OGG1* gene, reduces the spontaneous mutation frequencies to Can^R down to the wild type level (Thomas *et al.*, 1997)

In order to investigate whether the over-expression of the *OGG1* gene by plasmid pYSB10 can have a similar decreasing effect on the frequency of spontaneous mutation to Can^R in NER deficient backgrounds, the following experiments were undertaken.

The *OGG1* gene was over-expressed and the frequency of spontaneous mutation was determined first in wild type and *ogg1* mutants transformed with plasmid pYSB10 to provide references for comparison. Then the same experiments were performed in the NER deficient *rad14* and *ogg1rad14* mutants harboring plasmid pYSB10 (constructed in chapter 2). The following sections firstly compare the frequency of spontaneous mutation to Can^R for each strain, before and after being transformed with plasmid pYSB10. A comparison will then be made between the effects of over-expressing the *OGG1* gene in different strains.

3.4.2.1 Over-expressing *OGG1* in the wild type

The over-expression of the *OGG1* gene did not have any significant effect on the frequency of spontaneous mutation to Can^R in the wild type (Scott *et al.*, 1999).

Table 3.10 summarizes the frequencies of Can^R mutants in the original wild type (FF1873) and in the wild type transformed by plasmid pYSB10. (FF19733 + pYSB10) in experiments 3.5-3.7. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of 3 independent experiments showing the same pattern of increase are illustrated graphically as bar charts in Fig.3.11. The raw data are contained in Appendix III.a (A III.a.5-A III.a.7).

	Can $^{R}/10^{6}$			
wild type	**pYSB10^{-}**		**pYSB10+**	
	Median	**(LIQV, UIQV)**	**Median**	**(LIQV, UIQV)**
Exp. 3.5	0.19	(0.18, 0.21)	0.24	(0.12, 0.27)
Exp. 3.6	0.24	(0.23, 0.26)	0.28	(0.24, 0.34)
Exp. 3.7	0.23	(0.23, 0.38)	0.25	(0.21, 0.26)

Table 3.10 The frequency of spontaneous mutation to Can^{R} in the wild type with and without plasmid pYSB10.

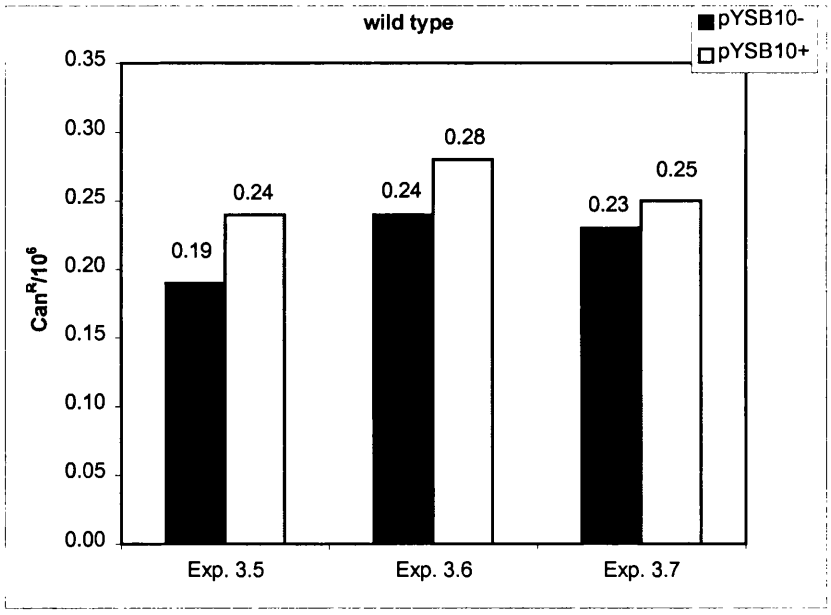


Fig. 3.11 Bar charts comparing the frequency of spontaneous mutation to Can^{R} in the wild type with and without plasmid pYSB10.

3.4.2.2 Over-expressing *OGG1* in the *ogg1* mutants

In accord with previous findings (Thomas *et al.*, 1997), transformation of the *ogg1* mutants with plasmid pYSB10 which over-expresses the *OGG1* gene, completely suppressed the enhanced frequency of spontaneous mutation to Can^R (Scott *et al.*, 1999). An average of an 18 fold decrease was observed in the *ogg1* mutants carrying the pYSB10 (*ogg1* + pYSB10) compared to the original *ogg1* mutant strain (CD138).

Table 3.11 summarizes the frequencies of Can^R mutants in the original *ogg1* mutant (CD138) and in the *ogg1* mutant transformed by plasmid pYSB10. (*ogg1* + pYSB10) in experiments 3.5-3.7. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of 3 independent experiments showing the same pattern of decrease are illustrated graphically as bar charts in Fig. 3.12. The raw data and statistical analysis are contained in Appendix III.a (A III.a.5-A III.a.7) and Appendix III.b (A III.b.8) respectively.

	Can $^{R}/10^{6}$			
ogg1	pYSB10-		pYSB10+	
	Median	**(LIQV, UIQV)**	**Median**	**(LIQV, UIQV)**
Exp. 3.5	1.17	(0.78, 1.47)	0.06	(0.05, 0.12)
Exp. 3.6	1.07	(0.71, 2.20)	0.18	(0.14, 0.21)
Exp. 3.7	2.61	(2.29, 2.65)	0.09	(0.08, 0.14)

Table 3.11 The frequency of spontaneous mutation to Can^{R} in the *ogg1* mutants with and without plasmid pYSB10.

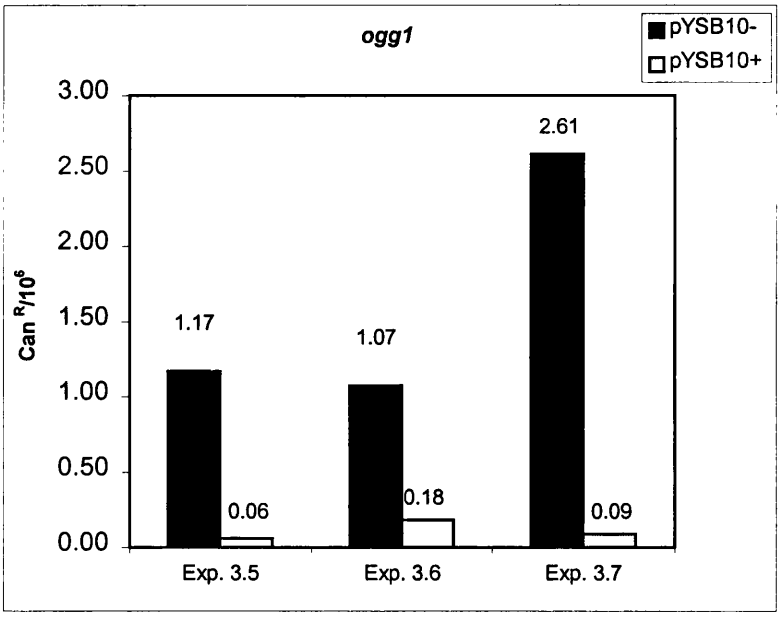


Fig. 3.12 Bar charts comparing the frequency of spontaneous mutation to Can^{R} in the *ogg1* mutants with and without plasmid pYSB10.

3.4.2.3 Over-expressing *OGG1* in the *rad14* mutant

In order to investigate whether the increase in spontaneous mutation due to the loss of NER can be compensated for by over-expression of the *OGG1* gene, the frequency of spontaneous mutation to Can^R was studied in a *rad14* mutant strain harboring plasmid pYSB10 .

The results of these experiments indicated no statistically significant difference in the frequency of spontaneous mutations to Can^R between the *rad14* mutant strain harboring plasmid pYSB10 and the original *rad14* strain (Scott *et al.*, 1999).

Table 3.12 summarizes the frequencies of Can^R mutants in the original *rad14* mutant (ADS100) and in the *rad14* mutant transformed by plasmid pYSB10 (*rad14* + pYSB10) compared in experiments 3.5-3.7. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of 3 independent experiments showing the same pattern of increase are illustrated graphically as bar charts in Fig. 3.13. The raw data and statistical analysis are contained in Appendix III.a (A III.a.5-A III.a.7) and Appendix III.b. (A III.b.9) respectively.

	Can $^{R}/10^{6}$			
rad14	pYSB10-		pYSB10+	
	Median	**(LIQV, UIQV)**	**Median**	**(LIQV, UIQV)**
Exp. 3.5	2.48	(2.41, 2.76)	2.02	(1.98, 2.26)
Exp. 3.6	2.34	(2.15, 2.50)	2.31	(2.31, 2.74)
Exp. 3.7	2.24	(2.20, 2.35)	2.28	(2.17, 2.43)

Table 3.12 The frequency of spontaneous mutation to Can^{R} in the *rad14* mutants with and without plasmid pYSB10.

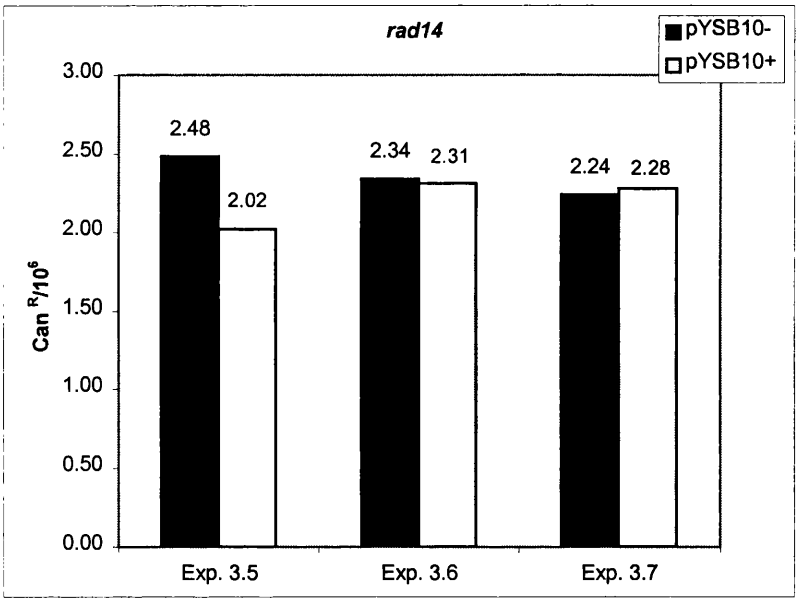


Fig. 3.13 Bar charts comparing the frequency of spontaneous mutation to Can^{R} in the *rad14* mutants with and without plasmid pYSB10.

3.4.2.4 Over-expressing *OGG1* in the *ogg1rad14* mutant

The results of the last experiments indicated that over-expression of *OGG1* gene does not significantly reduce the frequency of spontaneous mutation to Can^R in the *rad14* strains. Here, experiments were performed to determine whether the complete loss of NER together with inactivation of BER can be compensated for by the over-expression of the *OGG1* gene. Thus, the frequency of spontaneous mutation to Can^R was determined in the *ogg1rad14* strain transformed with plasmid pYSB10.

There was a slight decrease in the frequency of Can^R mutation between the *ogg1rad14* mutant harboring plasmid pYSB10 and the original *ogg1rad14* mutant strain but this decrease was not statistically significant (Scott et al., 1999).

Table 3.13 summarizes the frequencies of Can^R mutants in the original *ogg1rad14* mutant (ADS110) and in the *ogg1rad14* mutant transformed by plasmid pYSB10 (*ogg1rad14* + pYSB10) compared in experiments 3.5-3.7. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The results of 3 independent experiments showing the same pattern of increase are illustrated graphically as bar charts in Fig. 3.14. The raw data and statistical analysis are contained in Appendix III.a (A III.a.5-A III.a.7) and Appendix III.b (A III.b.10) respectively.

	Can $^{R}/10^{6}$			
ogg1rad14	pYSB10-		pYSB10+	
	Median	**LIQV, UIQV**	**Median**	**(LIQV, UIQV)**
Exp. 3.5	2.61	(2.23, 3.28)	1.8	(1.31, 2.00)
Exp. 3.6	1.96	(1.79, 2.13)	1.26	(1.19, 1.27)
Exp. 3.7	1.4	(1.31, 1.65)	0.69	(0.68, 0.73)

Table 3.13 The frequency of spontaneous mutation to Can^{R} in the *ogg1rad14* mutants with and without plasmid pYSB10.

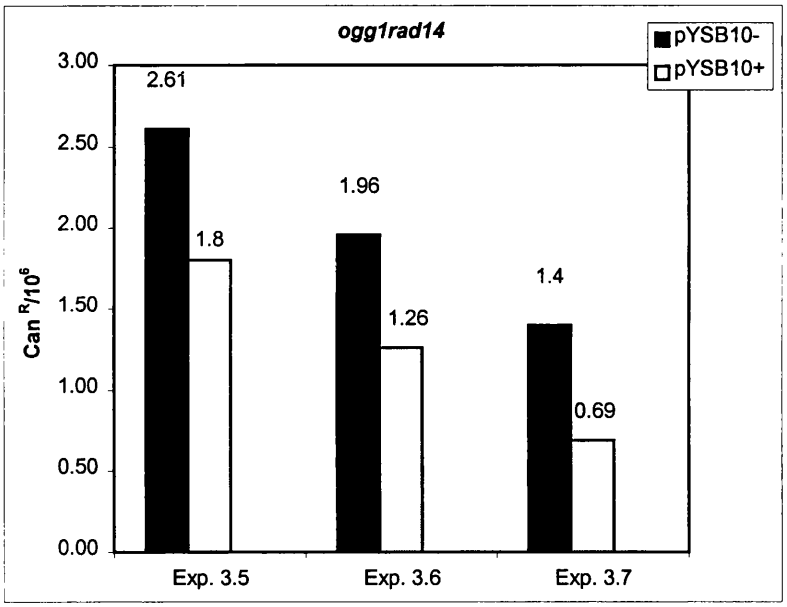


Fig. 3.14 Bar charts comparing the frequency of spontaneous mutation to Can^{R} in the *ogg1rad14* with and without plasmid pYSB10.

In each of experiments 3.5-3.7, all 8 strains (wild type, *ogg1, rad14, ogg1rad14*, and their corresponding pYSB10 harboring strains) were examined under the same experimental conditions except for the strain specific amino acid supplement to the minimal media. Tables 3.14 to 3.16 compare the frequencies of Can^R mutants in the wild type (FF1873), *ogg1* (CD138), *rad14* (ADS100) and *ogg1rad14*(ADS110) in relation to their pYSB10 transformed counterparts in experiments 3.5-3.7. The frequency of the spontaneous mutation to Can^R is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. These experiments are demonstrated graphically as bar charts in Fig. 3.15 to 3.17.

Exp. 3.5

	can^R/10^6				
	pYSB10-		pYSB10+		
	Median	(LIQV,UIQV)	Median	(LIQV, UIQV)	pYSB10-/pYSB10+
WILD	0.19	(0.18, 0.21)	0.24	(0.12, 0.27)	1
ogg1	1.17	(0.78, 1.47)	0.06	(0.05, 0.12)	20
rad14	2.48	(2.41, 2,76)	2.02	(1.98, 2.26)	1
ogg1rad14	2.61	(2.23, 3.28)	1.80	(1.31, 2)	1

Table 3.14 The effect of overexpression of the *OGG1* gene by plasmid pYSB10, on the frequency of spontaneous mutation to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* strains of *S.cerevisiae* .

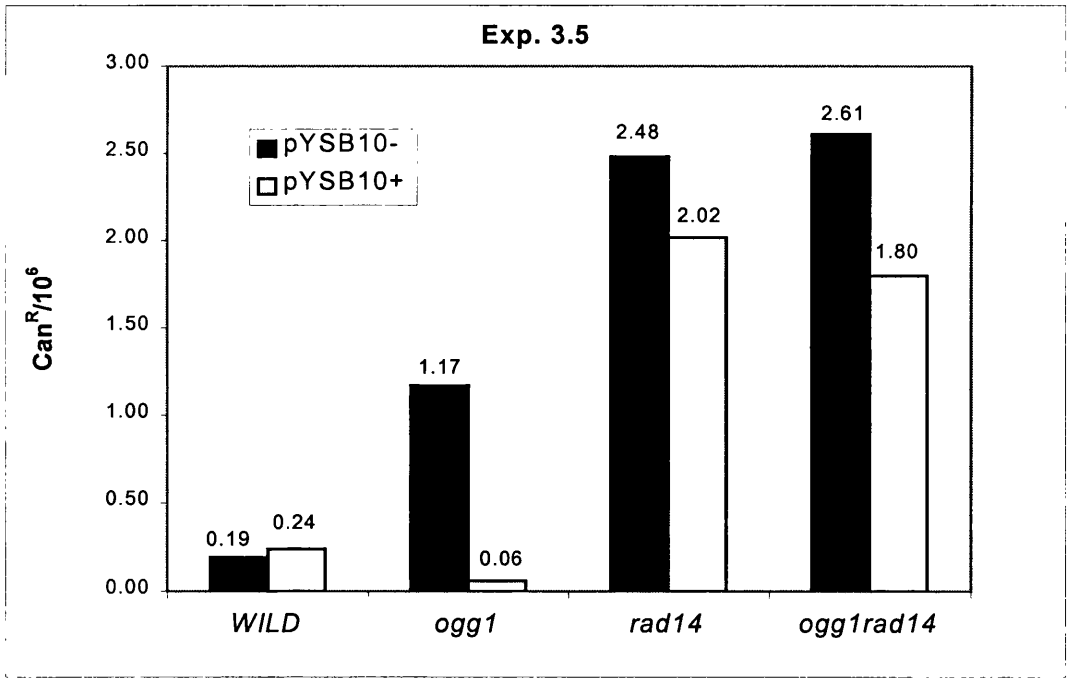


Fig. 3.15 Bar charts comparing the effect of the overexpression of the *OGG1* gene by plasmid pYSB10 on the frequency of spontaneous mutations to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* mutants of *S.cerevisiae.*

Exp. 3.6

	can^R/10^6				
	pYSB10-		pYSB10+		
	Median	(LIQV,UIQV)	Median	(LIQV, UIQV)	pYSB10-/pYSB10+
WILD	0.24	(0.23, 0.26)	0.28	(0.24, 0.34)	1
ogg1	1.07	(0.71, 2.20)	0.18	(0.14, 0.21)	6
rad14	2.34	(2.15, 2.50)	2.31	(2.31, 2.74)	1
ogg1rad14	1.96	(1.79, 2.13)	1.26	(1.19, 1.27)	2

Table 3.15 The effect of overexpression of the *OGG1* gene by plasmid pYSB10, on the frequency of spontaneous mutation to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* strains of *S.cerevisiae* .

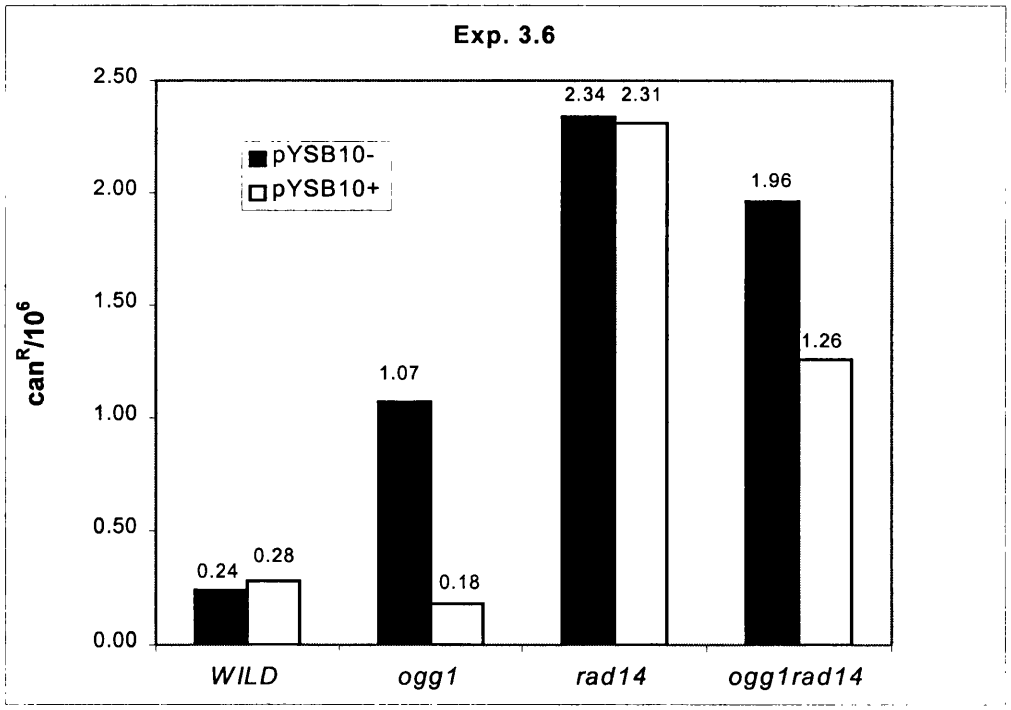


Fig. 3.16 Bar charts comparing the effect of the overexpression of the *OGG1* gene by plasmid pYSB10 on the frequency of spontaneous mutations to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* mutants of *S.cerevisiae.*

Exp. 3.7

	can^R/10^6				
	pYSB10-		pYSB10+		
	Median	(LIQV,UIQV)	Median	(LIQV, UIQV)	pYSB10-/pYSB10+
WILD	0.23	(0.23, 0.38)	0.25	(0.21, 0.26)	1
ogg1	2.61	(2.29, 2.65)	0.09	(0.08, 0.14)	29
rad14	2.24	(2.20, 2.35)	2.28	(2.17, 2.43)	1
ogg1rad14	1.4	(1.31, 1.65)	0.69	(0.68, 0.73)	2

Table 3.16 The effect of overexpression of the *OGG1* gene by plasmid pYSB10, on the frequency of spontaneous mutation to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* strains of *S.cerevisiae* .

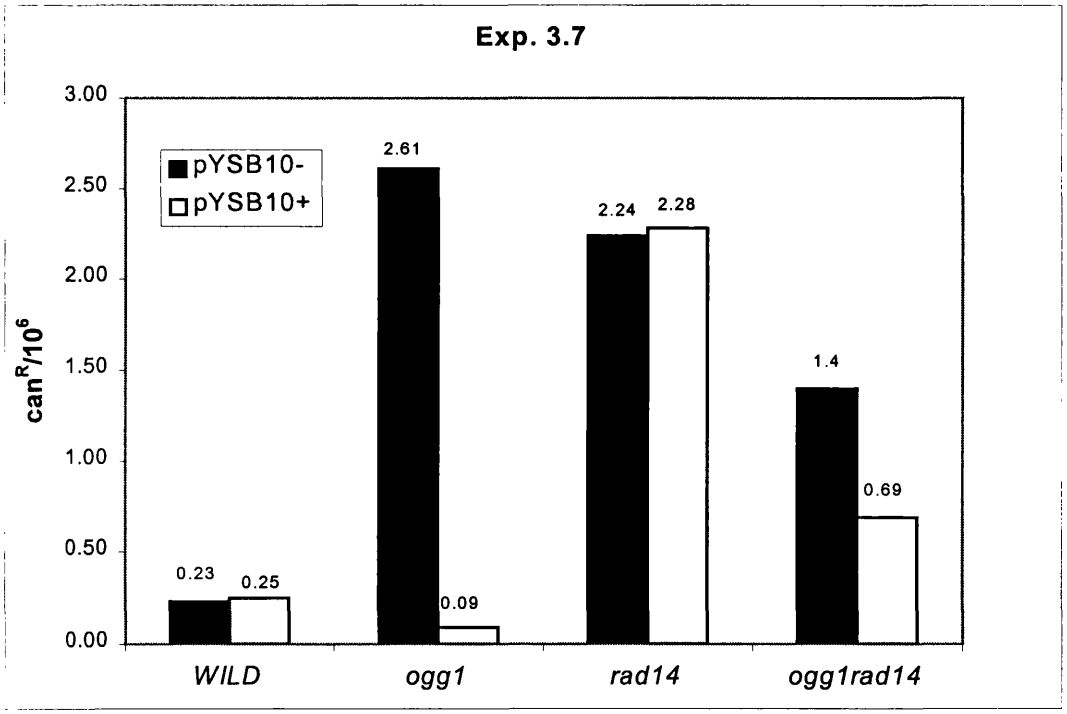


Fig. 3.17 Bar charts comparing the effect of the overexpression of the *OGG1* gene by plasmid pYSB10 on the frequency of spontaneous mutations to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* mutants of *S.cerevisiae.*

3.4.3 Northern blot analysis : over- expression of the plasmid borne *OGG1* gene

In order to confirm that the earlier results are not due to the inefficient expression of the *OGG1* gene by plasmid pYSB10 in some of the strains, the total RNA was extracted from these strains and a northern hybridization was performed using a radio-labeled *OGG1* probe and a radio-labeled *URA3* probe as control.

Fig. 3.19 represents the northern blot of the total RNA derived from different strains probed with a ^{32}P labeled *OGG1* probe in A and a ^{32}P labeled *URA3* probe in B.

Lane1 represents total RNA from the *ogg1rad14* strain, which does not carry the plasmid pYSB10. *OGG1* is not expressed in this lane (A.lane1). Lanes 2-5 represent total RNA derived from wild type, *ogg1, rad14*, and *ogg1rad14* strains respectively, all harboring plasmid pYSB10. Clearly, *OGG1* is expressed in high quantities in all these strains (A. lane2-5). *URA3,* as positive control is expressed in all strains (B. Lanes 1-5).

These results indicate that the *OGG1* gene is over-expressed efficiently in the wild type, *ogg1, rad14* and *ogg1rad14* strains harboring pYSB10. Thus, the differences observed between the result of the Can^R assay between these strains can not be due to the plasmid pYSB10 failing to over-express the *OGG1* gene sufficiently in these transformants.

A

B

Figure 0. Northern blot of total RNA ($100_{\mu g}$/lane) extracted from 1) *ogg1rad14*, 2)wild type + pYSB10, 3) *ogg1*+ pYSB10, 4) *rad14* + pYSB10, and 5) *ogg1rad14* + pYSB10. (A): Total RNA blotted on the membrane is probed with the radio-labelled *OGG1* probe. (B): The Same membrane as (A) which is probed with *URA3* probe.

3.5 Discussion

The canavanine forward mutation assay allows one to examine how the failure of DNA repair mechanisms influences spontaneous mutation. Consistent with the data of Thomas *et al.*, (1997), the *ogg1* mutant demonstrated a significant increase in the frequency of spontaneous mutations to Can^R relative to the wild type. Disruption of the *RAD14* gene in the wild type strain also gave rise to a significant increase in the frequency of spontaneous mutation to Can^R. This increase was similar to the levels obtained in the *ogg1* mutant. Likewise, disruption of the *RAD14* gene in an *OGG1* deficient background, led to a significant increase in Can^R mutation relative to the wild type. However, no additional increase in the frequency of spontaneous mutations to Can^R was detected in the *ogg1rad14* mutants relative to either the *rad14* or the *ogg1* single mutants (Scott *et al.*, 1999).

Previously, it had been shown that over-expression of the *OGG1* gene completely suppresses the frequency of spontaneous mutations to Can^R to the wild type level (Thomas *et al.*, 1997). Using the same approach, the effect of over-expression of the *OGG1* gene on the frequency of spontaneous mutations to Can^R was studied in the wild type, *ogg1*, *rad14* and *ogg1rad14* strains transformed with a high copy number plasmid pYSB10, which over-expressed the *OGG1* gene. In accord with previous findings (Thomas *et al.,* 1997), over-expression of the *OGG1* gene in the *ogg1* mutant suppressed the frequency of spontaneous mutation to Can^R down to the wild type level. However, the over-expression of the *OGG1* did not significantly decrease the

frequency of Can^R mutants in *rad14* . Similarly, although there was a slight reduction in the Can^R mutation frequency in the *ogg1rad14* harbouring plasmid pYSB10 compared to the original *ogg1rad14*, this decrease was not statistically significant (Scott *et al.*, 1999). Northern hybridisation confirmed that the differences in the results between these strains were not due to an inefficient over-expression of the *OGG1* gene in some of the mutants.

These data once again support the role of the *OGG1* gene in the repair of damages leading to spontaneous mutations to Can^R , but they also indicate that *OGG1* alone, whether it is expressed in normal amounts or over-expressed via pYSB10 high copy number plasmid, cannot compensate for the loss of NER activity so as to reduce the frequency of spontaneous mutation to Can^R. The high level of Can^R mutations in the *ogg1* mutant is likely due to a specific GC to TA mutation. A number of other base damages may give rise to forward mutation to Can^R. Therefore, these results indicate that NER has certainly a role in removing some base lesions leading to forward mutation to Can^R. The nature of these lesions is unknown. The high level of spontaneous mutation to Can^R in the *ogg1* or *rad14* single mutants relative to the wild type and the fact that by over-expression of plasmid pYSB10, the frequency of spontaneous mutations to Can^R was not significantly reduced in the *rad14* and only slightly reduced in the *ogg1rad14* strain (if despite the statistical insignificance, the slight reduction is biologically significant), suggests that NER and BER may have different substrate specificities for lesions leading to mutation. However, if this is the case, why wasn't spontaneous mutation rate higher in the *ogg1rad14* double mutant compared to either single mutant? Possibly, there is an interaction between NER and BER proteins, which is necessary for controlling the spontaneous mutations in

S.cerevisiae. This would also explain the absence of a statistically significant decrease in spontaneous mutation of the *ogg1rad14* double mutant by over-expression of the *OGG1* gene (if the slight reduction has no biological significance). The recent finding that hNth1, which is the human homologue of endonuclease III or Nth in *E.coli* functions more efficiently with NER 3' endonuclease XPG protein (Bessho *et al.*, 1999; Klungland *et al.*, 1999), indicates that there may be certain interactions between NER and BER proteins that influences the ability to interpret the data in this chapter.

Chapter 4

Spontaneous GC to TA transversions in *rad14* mutants of *S.cerevisiae*

4.1 Introduction

The experiments in the previous chapter examined the role of NER and BER in spontaneous forward mutation at the arginine permease locus. The results indicated a role for both pathways in controlling spontaneous forward mutation. However, forward mutation of a wild type gene to a mutant type can involve a large number of different unbiased mutational events, and it gives no indication of the exact nature and position of the lesions causing that mutation.

A more precise approach to examine the contributions of NER and BER to control spontaneous mutations, necessitates a knowledge of the exact nature and the position of the base modifications that give rise to a particular spontaneous mutation. Since a very limited number of biased mutations can lead to the reversion of a given mutant phenotype back to the wild type, reversion mutations can offer convenient assays to detect a few specific events at a few specific sites (Friedberg *et al.,* 1995).

A Lys^+ reversion system was employed by Thomas *et al.*, (1997) who demonstrated that the frequency of spontaneous mutation to Lys^+ was significantly increased in an

ogg1 strain compared to the wild type. Using the same assay, recently it has been shown that disruption of the *RAD14* gene in either the wild type or an *OGG1* deficient background does not significantly increase the spontaneous mutations to Lys^+ (Scott *et al.*, 1999). These results are in contrary to the previous findings for forward mutation, which suggested a role for NER in this process. This contradiction can be explained as follows: It has been argued that, since the *ogg1* mutant is a specific GC to TA mutator, the increase in Lys^+ revertants most probably arises as a result of a specific GC to TA mutational event. Nevertheless the *lys1-1* ochre allele does not contain any potential sites for intragenic GC to TA mutational events. This suggests that reversion to Lys^+ could be due to an external information suppressor mutation, as reported for a *rad18* mutant, which is also a GC to TA mutator (Kunz *et al.*, 1991; Cassier- Chauvat and Fabre 1991). Supporting this hypothesis, a specific GC to TA base substitution event in a tRNA ser gene anticodon TGA, was shown to lead to the suppression of the *lys1-1* ochre allele (Scott *et al.*, 1999). Several possible interpretations have been made based on this assumption.

Firstly, if NER and BER have different affinities for lesions based on their genomic locations, then even if *OGG1* and *RAD14* proteins compete for the similar substrates, the disruption of the *RAD14* gene will not necessarily affect the spontaneous mutations to Lys^+. In other words, *OGG1* and *RAD14* may both be involved in removing 8-oxoG lesions from DNA but some other events such as the assembly of regulatory factor TFIIIC and RNA polymerase III at promoter elements of tRNA genes, may block the accessibility of a NER repair complex to 8-oxoG lesions in these genomic locations. As a result, the role of NER in removing 8-oxoG depends on the location of the lesion.

Another interpretation is based on the fact that enhanced repair of transcribed strands is due to the preferential repair of RNA polymerase II transcribed genes (Mellon and Hanawalt, 1989). Certain NER gene products are subunits of transcription factor IIH, (TFIIH), a component of the basal transcription initiation machinery (Van Good *et al.*, 1994; Troelstra *et al.*, 1992). In contrast, no transcription-coupled repair has been observed for tRNA or rRNA genes in humans, which are transcribed by RNA polymerase III and I respectively (Christian and Hanawalt, 1993; Fritz and Smerdon 1995; Dammann and Pfeifer, 1997). In *S.cerevisiae*, it has been shown that, although the repair of rRNA genes can be strand specific and coupled to transcription (Verhage *et al.*, 1996c), the transcription coupled repair of CPDs does not take place in a serine tRNA gene which is transcribed by RNA polymerase III. Based on the above, no increase in spontaneous mutation to Lys^+ can be explained if NER is coupled to RNA polymerase II transcribed genes at the expense of inefficient NER at loci transcribed by other RNA polymerases. In other words, the role of NER in the repair of 8-oxoG might be insignificant in those regions of the genome not being transcribed by RNA polymerase II (Scott *et al.*, 1999).

The third possible interpretation is that 8-oxoG may not be a substrate for NER, a question, which remains to be answered by further experimentation (Scott *et al.*, 1999).

4.2 The present study

The experiments presented next in this chapter further investigate the roles of NER and BER in the repair of oxidative base damage by using another reversion system

that can specifically detect GC to TA mutations. The Cyc^+ reversion system developed by Hampsey *et al.*, (1991) has been previously used to analyse the spectrum of base substitutions in *S.cerevisiae*. Using this system it has been demonstrated that the *ogg1* deficient strain is a specific GC to TA mutator; suggesting that 8-oxoG lesions in double stranded DNA represents a substrate for Ogg1 *in vivo*. To determine whether these residues are a substrate for NER, the Cyc^+ reversion system was employed to investigate the frequency of GC to TA transversions in NER deficient strains of *S.cerevisiae* constructed as described in chapter 2, by disruption of the *RAD14* gene in a wild type and in an *ogg1* deficient background. Unlike the Lys^+ reversion assay, the Cyc^+ reversion assay, designed by Hampsey *et al* (1991) has the advantage of offering an intragenic location for GC to TA mutational events.

4.3 Materials and Methods

4.3.1 The Hampsey strains

The reporter system designed by Hampsey *et al.*, (1991) facilitates the detection of all 6 possible base pair substitution events (table 4.1). This system employs a group of isogenic *S.cerevisiae* strains (YMH2-7). The only difference among these strains is a single base substitution within the cycteine 22 codon of the *CYC1* gene encoding iso-1 cytochrome c. As a result of this single base substitution, these Cyc^- mutant strains are unable to grow on a nonfermentable carbon source such as medium containing glycerol as the sole carbon source.

A reverse mutation at the site of the single base substitution, by another specific single base substitution event can restore the normal base sequence and the Cyc^+ phenotype in each of these tester strains, enabling them to grow on medium containing glycerol as the sole carbon source. Therefore, the frequency of Cyc^+ revertants growing on glycerol medium is indicative of a specific base substitution event. The Hampsey strains and their corresponding substrate specificities are presented in table 4.1. In the experiments presented in this chapter, the YMH-4 which is a specific GC → TA reporter system and it's *rad14* or/and *ogg1* deficient derivatives were used.

Haploid strain	Codon 21-23	Amino acid 22	Mutational specificity
YMH1	CAA TGC CAC	Cys	
YMH2	CAA CGC CAC	Arg	G.C → A.T
YMH3	CAA AGC CAC	Ser	A.T → T.A
YMH4	**CAA GGC CAC**	**Gly**	**G.C → T.A**
YMH5	CAA TCC CAC	Ser	G.C → C.G
YMH6	CAA TTC CAC	Phe	A.T → C.G
YMH7	CAA TAC CAC	Tyr	A.T → G.C

Table 4.1 DNA sequence changes in codon –22 are underlined. Codons 21 and 23 are included to depict the immediate sequence context flanking the mutational targets. The strain YMH-4 used in this study is highlighted (*Adapted from Hampsey et al., 1991*).

4. 3.2 *S.cerevisiae* strains used in this study

Table 4.2 summarises the *S.cerevisiae* strains used in this chapter. The YOG4 strain is derived from the YMH4 wild type strain by disruption of the *OGG1* gene. The ADS304 and ADS314 have been constructed by disruption of the *RAD14* gene as described in chapter 2, in the wild type YMH4 and the *ogg1*-deficient YOG4 strains respectively.

Strain	Description
YMH4	Wild type
YOG4	*ogg1*
ADS304	*rad14*
ADS314	*ogg1rad14*

Table 4.2 The *S.cerevisiae* strains used in this study

4.3.3 GC to TA base substitution analysis

Cells were grown in 25ml YPD at 28°C to a density of ~ 1.0 x 10^8 cells/ml. Cultures were obtained by incubation at an initial density of 2.5 x 10^4 cells/ml. Cell density was measured by plating 0.1ml dilutions on YPD plates, and were scored after incubation for 3 days at 28°C . To quantify the number of Cyc^+ revertants, cultures were concentrated 10-100 fold and 0.1 ml was plated on to YPG complete medium containing 3% glycerol as the sole carbon source. YPG plates were scored after incubation for 10 days at 28°C. The frequency of spontaneous mutation was calculated according to the following formula:

Mutation frequency = a / b

Where:

a = the average number of colonies on the YPG plate divided by concentration factor.

b = the average number of colonies on the YPD plate multiplied by dilution factor

4.4 Results

4.4.1 The frequency of spontaneous GC to TA mutations in the *ogg1* deficient and *rad14* deficient strains of *S.cerevisiae*

In accord with the findings of Thomas *et al.*, 1997, the *ogg1* mutant exhibited a significant increase in the frequency of GC to TA transversions relative to the wild type (Scott *et al.,* 1999): an average increase of a 7 fold was observed in the frequency of Cyc^+ reversions in the *ogg1* mutant.

Disruption of the *RAD14* gene in the wild type did not give rise to a significant increase in the frequency of spontaneous GC to TA mutations i.e. no significant difference was detected between the frequency of Cyc^+ revertants in the *rad14* mutant relative to the wild type (Scott *et al.*, 1999).

Table 4.1.a- 4.4.a summarise the frequencies of the of Cyc+ reversions in the wild type, *ogg1* and *rad14* strains of *S.cerevisiae* in experiments 4.1-4.4 respectively. The frequency of spontaneous mutation to Cyc^+ is represented as the median, lower and upper inter-quartile values of at least 5 independent colonies. The same pattern has been repeated in 4 independent experiments, and is illustrated graphically in Fig. 4.1.a to 4.4.a The raw data are contained in Appendix IV.a (A IV.a.1- A IV.a.4).

Expt. 4.1	Cyc$^+$/10^7		
	Median	**LIQV**	**UIQV**
wild type	0.02	0.02	0.02
ogg1	0.1	0.08	0.12
rad14	0.02	0.02	0.02

Table 4.1.a The frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *rad14* strains compared to the wild type.

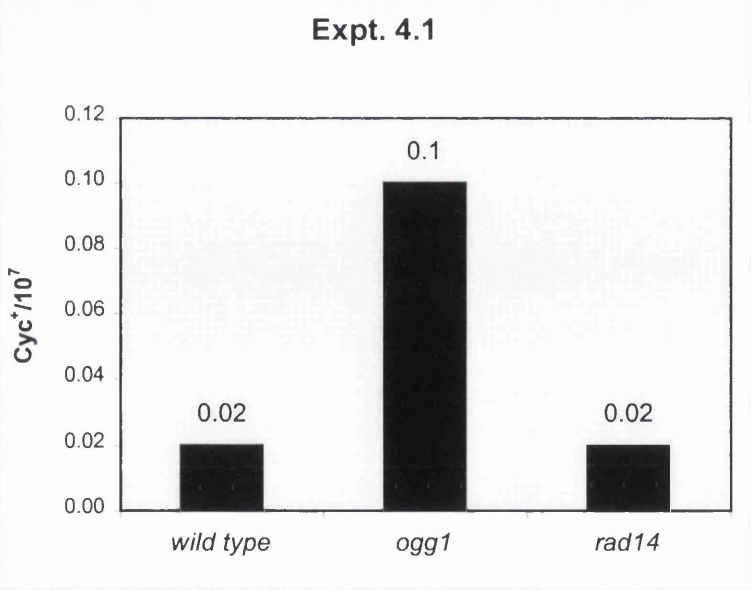


Fig. 4.1.a Bar charts comparing the frequency of spontaneous reversion to Cyc$^+$ between the wild type, *ogg1* and the *rad14* strains.

Expt. 4.2	Cyc$^+$/10^7		
	Median	**LIQV**	**UIQV**
wild type	0.04	0.03	0.04
ogg1	0.37	0.12	0.65
rad14	0.05	0.02	0.05

Table 4.2.a The frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *rad14* strains compared to the wild type.

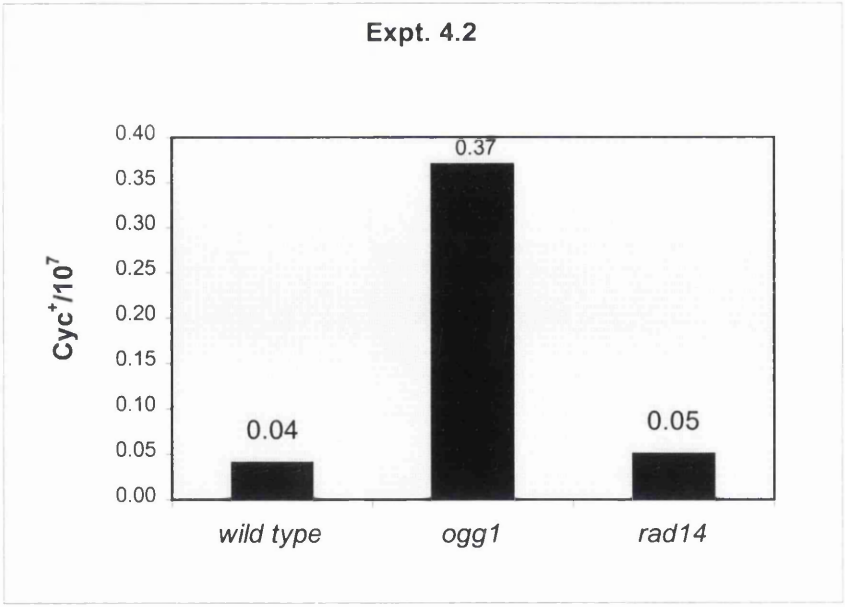


Fig. 4.2.a Bar charts comparing the frequency of spontaneous reversion to Cyc$^+$ between the wild type, *ogg1* and the *rad14* strains.

Expt. 4.3	Cyc$^+$/10^7		
	Median	**LIQV**	**UIQV**
wild type	0.05	0.05	0.05
ogg1	0.5	0.34	0.71
rad14	0.05	0.04	0.05

Table 4.3.a The frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *rad14* strains compared to the wild type.

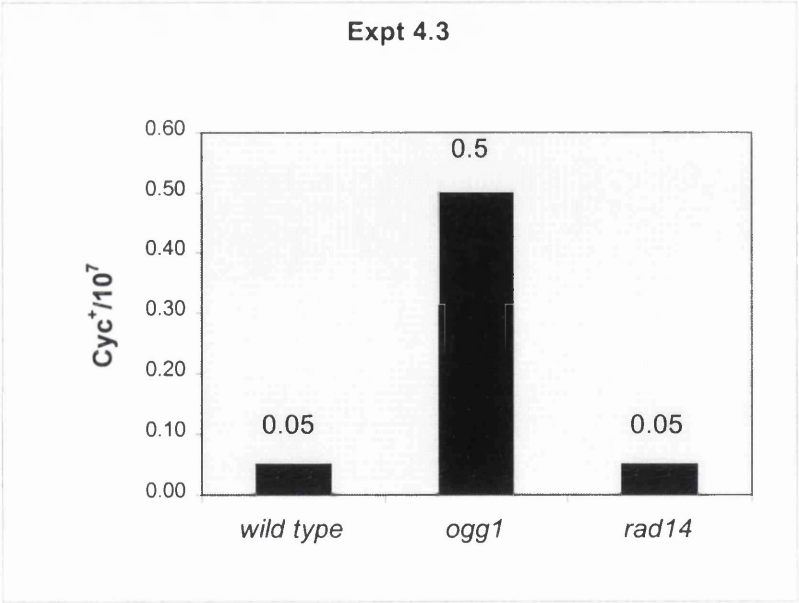


Fig. 4.3.a Bar charts comparing the frequency of spontaneous reversion to Cyc$^+$ between the wild type, *ogg1* and the *rad14* strains.

Expt. 4.4	$Cyc^+/10^7$		
	Median	**LIQV**	**UIQV**
wild type	0.02	0.02	0.02
ogg1	0.1	0.06	0.17
rad14	0.02	0.02	0.02

Table 4.4.a The frequency of spontaneous reversion to Cyc^+ in the *ogg1* and *rad14* strains compared to the wild type.

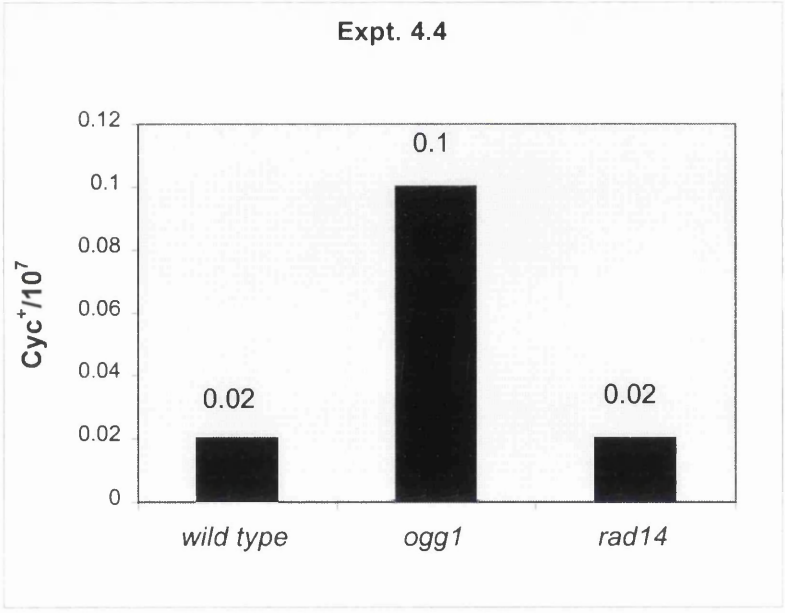


Fig. 4.4.a Bar charts comparing the frequency of spontaneous reversion to Cyc^+ between the wild type, *ogg1* and the *rad14* strains.

4.4.2 The frequency of spontaneous GC to TA transversion in the *ogg1rad14* strain compared to the *ogg1* strain

The *ogg1rad14* strain showed a significant increase in the frequency of spontaneous GC to TA transversions compared to the single *ogg1* mutant (Scott *et al.*, 1999). An average of a 21 fold increase was observed in the frequency of CYC^+ reversions for the *ogg1rad14* strain compared to the *ogg1* mutant.

Table 4.1.b – 4.2.b summarise the frequency of Cyc^+ reversions in the *ogg1* and *ogg1rad14* strains of *S.cerevisiae* in experiments 4.1-4.4 respectively. The frequency of spontaneous mutation to Cyc^+ is represented as the median, upper and lower inter-quartile values of at least 8 independent colonies. The results of 4 independent experiments representing the same trend are illustrated graphically as bar charts in Figs. 4.1.b-4.2.b The raw data are contained in Appendix IV.a (A IV.a.1-A IV.a.4). The statistical analysis for this data are contained in appendix IV.b (A IV.b.1-A IV.b.4).

Expt. 4.1	Cyc$^+$/10^7		
	Median	LIQV	UIQV
ogg1	0.1	0.08	0.12
ogg1rad14	2.94	0.44	3.79

Table 4.1.b The frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *ogg1rad14* mutants.

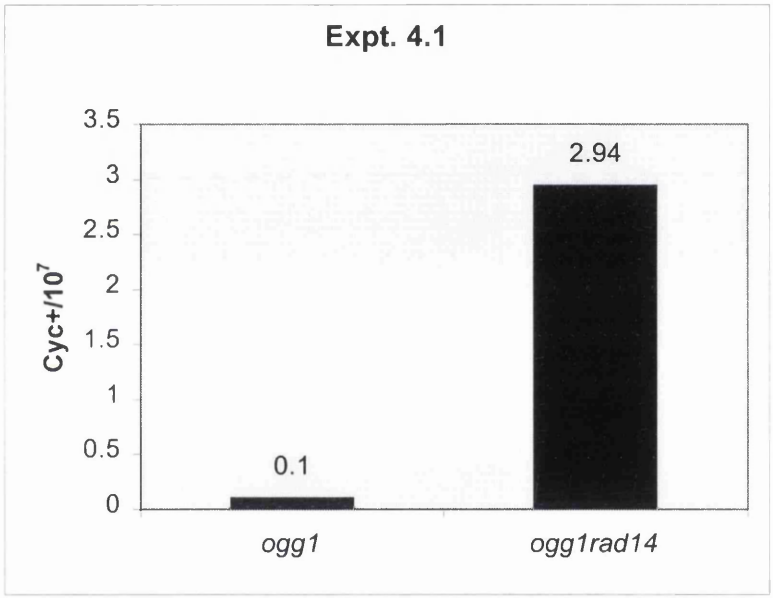


Fig 4.1.b Bar charts comparing the frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *ogg1rad14* mutants.

Expt. 4.2	Cyc$^+$/10^7		
	Median	LIQV	UIQV
ogg1	0.37	0.12	0.65
ogg1rad14	12.85	5.86	141.67

Table 4.2.b The frequency of spontaneous reversion to Cyc+ in the *ogg1* and *ogg1rad14* mutants.

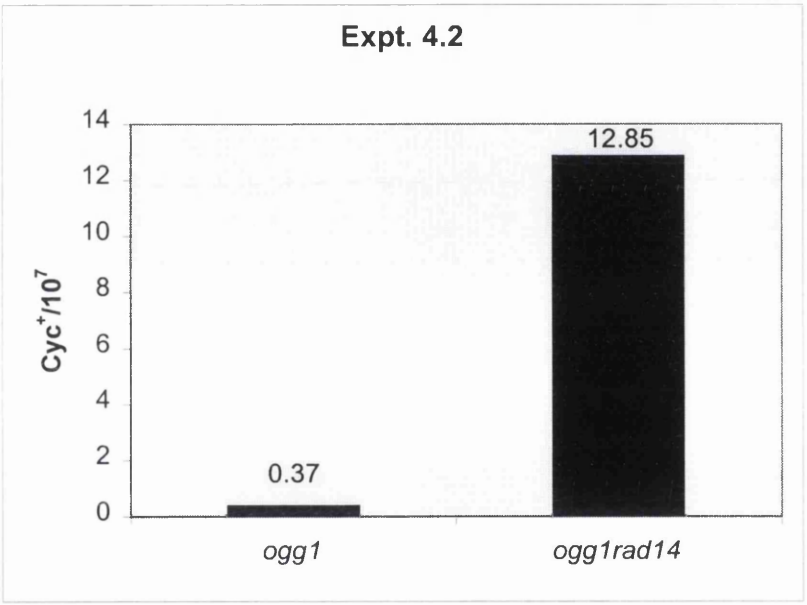


Fig 4.2.b Bar charts comparing the frequency of spontaneous reversion to Cyc$^+$ in the *ogg1* and *ogg1rad14* mutants.

Expt. 4.3	Median	$Cyc^+/10^7$ LIQV	UIQV
ogg1	0.5	0.34	0.71
ogg1rad14	6.58	1.34	28.04

Table 4.3.b The frequency of spontaneous reversion to Cyc+ in the *ogg1* and *ogg1rad14* mutants.

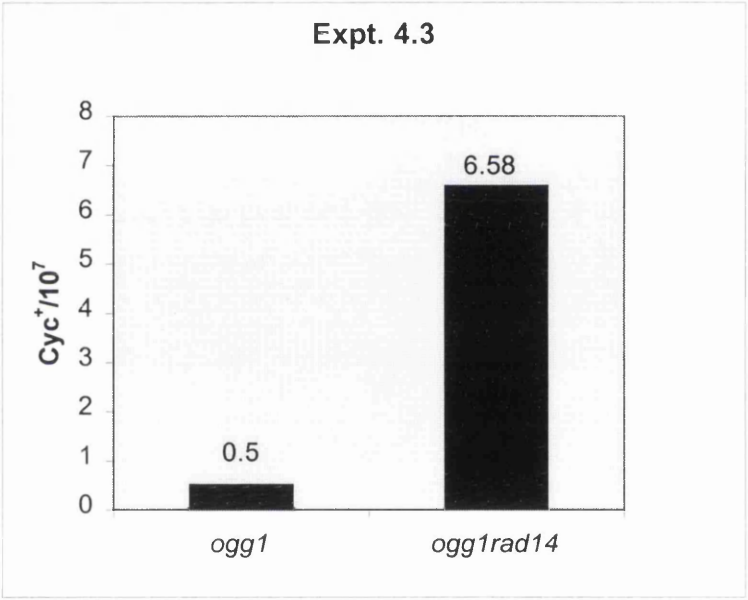


Fig 4.3.b Bar charts comparing the frequency of spontaneous reversion to Cyc^+ in the *ogg1* and *ogg1rad14* mutants.

Expt. 4.4	Median	$Cyc^+/10^7$ LIQV	UIQV
ogg1	0.1	0.06	0.17
ogg1rad14	0.58	0.19	5.12

Table 4.4.b The frequency of spontaneous reversion to Cyc+ in the *ogg1* and *ogg1rad14* mutants.

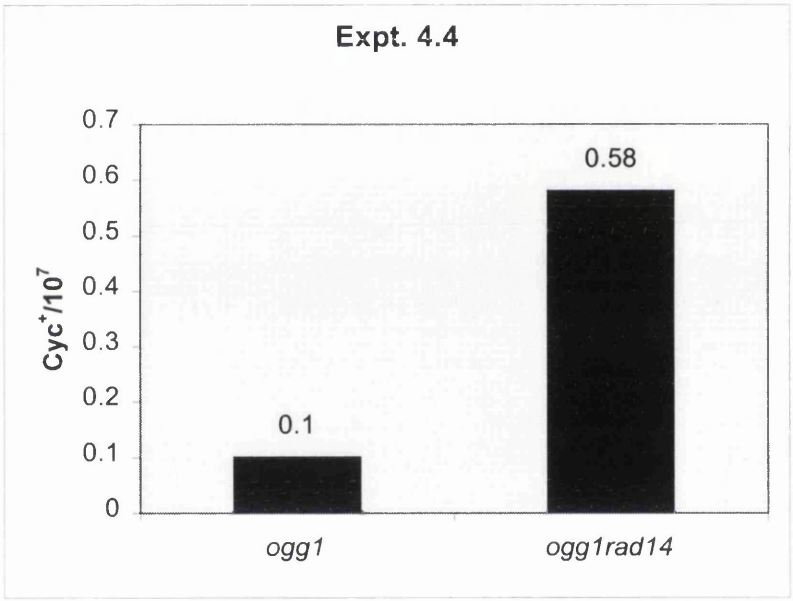


Fig 4.4.b Bar charts comparing the frequency of spontaneous reversion to Cyc^+ in the *ogg1* and *ogg1rad14* mutants.

4.5 Discussion

The role of the *OGG1* gene in the repair of 8-oxoG lesions has been demonstrated previously (Thomas *et al.*, 1997). In order to determine whether NER has also a significant contribution to the repair of 8-oxoG lesions leading to GC to TA transversions in *S.cerevisiae,* the Cyc^+ reversion system developed by Hampsey *et al.,* (1991), was used to investigate the frequency of specific GC to TA transversions in NER deficient strains. In support of the previous findings, the *ogg1* mutants exhibited a significant increase in the frequency of spontaneous Cyc^+ reversions relative to the wild type. However, the frequency of spontaneous mutations to Cyc^+ in *rad14* mutants was not significantly different with that of the wild type (Scott *et al.*, 1999). This suggests that the principle mechanism to remove 8–oxoG from DNA is base excision repair via *Ogg1*; and NER either has no significant role, or it's role in removing 8-oxoG lesions is being masked by the presence of *Ogg1*.

Interestingly, the *ogg1rad14* strains exhibited an additional significant increase in the frequency of spontaneous Cyc^+ reversions relative to the single *ogg1* mutant (Scott *et al.*, 1999). This suggests that *RAD14* has a role in the removal of 8-oxoG lesions from DNA, in the absence of Ogg1. These issues will be discussed further in the general discussion chapter.

Chapter 5

5.1 Introduction

Photosensitzer induced Fpg sensitive sites at nucleotide resolution in the nuclear DNA of *S.cerevisiae*

Very few specific oxidative base modifications can be detected by spontaneous mutational assays. These assays can not always define the nature of the mutational events and are unable to determine the relative position and distribution pattern of mutagenic lesions in the genome. An ideal system to study the role of NER/BER repair activities in the repair of oxidative base damages would be an assay, which allows us to view the effect of the disruption of these mechanisms at nucleotide resolution. Thus, one can specifically examine a spectrum of oxidative base lesions together with their relative position in the genome in relation to chromatin structure and transcription.

Such a system has been developed by Li and Waters (1996) in *E.coli* and Teng *et al.*, (1997) in *S.cerevisiae.* These papers described a method to detect UV-induced cyclobutane pyrimidine dimers (CPDs) and their repair at nucleotide resolution in DNA. In this assay the DNA fragment under the study is cut at specific damage sites

by a DNA repair enzyme. The TS and NTS are separated using biotinylated probes that are complementary to the TS or NTS respectively. The DNA fragments are then end-labeled by using sequenase enzyme to add radioactive nucleotides at the 3' end of the strand. This is followed by denaturing gel electrophoresis, which separates the fragments by their size and allows us to visualize the exact nucleotide position of the damage. In yeast, this technique first examined the *MFA2* gene, which codes for a2 factor involved in the pheromone response pathway (Kurjan *et al.*, 1993). When the work began, (before the whole genome had been sequenced) the sequence of *MFA2* was available (Mallet *et al.*, 1995) and its transcription control was well understood. The control and coding region of *MFA2* can be obtained from a 689 bp RsaI restriction fragment and the whole gene and its control region can be examined on one sequencing gel. These features made this gene an ideal model for examining DNA damage and repair at the level of the nucleotide (Teng *et al.*, 1997; Teng *et al.*, 1998).

On the basis of this, using the method described to examine CPDs in *MFA2*, the technique has been adapted and modified to examine oxidative DNA damage at the nucleotide resolution in *MFA2* (a nuclear gene) and in *OLI1*(a mitochondrial gene) (Meniel and Waters, 1999). Oxidative DNA damage can now be observed at nucleotide level in untreated DNA. In order to develop the assay, the DNA was exposed to the commonly used oxidative DNA damage inducer methylene blue with light (MB-light). MB is a thiazine dye, and a photosensitizer and the types of damages induced in the presence of light are well documented. These damages are 8-oxoG and fapy-G with 8-oxoG being induced 20 times more than fapy-G (Floyd *et al.*, 1990a; Boiteux *et al.*, 1992). MB does not cause detectable level of oxidative damage *in vivo;* so it could only be used *in vitro* for assay development (Meniel and Waters, 1999).

In order to develop the assay for the detection of oxidative base damage induced *in vivo*, a chemical synthesized by Hoffman la Roche; [R]-1[(10Chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol or RO 19-8022, referred to as RO has been used. This polycyclic aromatic can induce oxidative DNA damage in the presence of light. It has been shown that RO-light treatment of yeast cells can induce single strand breaks (SSB) and Fpg sensitive sites (FpgSS) in DNA, suggesting that either the chemical itself, or ROS produced outside of the cell in the presence of light, enter yeast (*The biological properties of the E.coli Fpg enzyme was discussed in detail in the general introduction chapter* [*1.3.2.2*]). No detectable formation of H_2O_2 has been reported outside the cells in presence of RO-light and an identical spectrum of SSB and FpgSS has been observed after RO-light treatment in the presence or absence of catalase, which catalyzes decomposition of H_2O_2 to molecular oxygen and water. Therefore, either the chemical itself enters yeast to produce oxidative base damage or it induces ROS outside the cells other than hydrogen peroxide. It has been shown that the majority of induced damages are base modifications, which are sensitive to the Fpg enzyme. High performance liquid chromatography (HPLC) analysis has demonstrated that as much as 74±10% of these Fpg sensitive sites are 8-oxoG (Pflaum *et al.*, 1997). After a 10 minutes of light, repair competent cells incubated with RO show a 10% survival with detectable spectrum of oxidative base damages. (Meniel and Waters, 1999). At best, a minimum of 10^6 copies of DNA can be detected by this assay, but $5x10^7$ copies are preferred for optimum results. The sensitivity of this assay has been estimated to be at least 0.2 SSB per 8.7kb for both nuclear and mitochondrial genomes (Meniel *et al.,* 1999) In the light of those data, the system was currently being optimized to monitor the repair

of oxidative base damages at nucleotide resolution (Meniel *et al.*, 1999). However, the high cytotoxicity of RO-light, was a major obstacle in detecting the repair at nucleotide resolution and RO chemical proved to be not a suitable chemical for induction of damage *in vivo* for monitoring DNA repair at nucleotide resolution (personal communications, Meniel, *et al.*, 2000).

5.2 The present study

The aim of the experiments in this chapter was to demonstrate the application of the 3' end labeling technique for its detection of *in vitro* and *in vivo* induced oxidative base damage at nucleotide resolution in the *MFA2* gene using MB-light and RO-light respectively (at the time of this experiment, RO was thought to be a suitable *in vivo* oxidative DNA damage inducing chemical for studying the repair of oxidative base damage).

5.3 Materials and Methods

5.3.1 *S.cerevisiae* strains

Yeast haploid W303 *rad14* (a *ade, his , leu,trp,* CAN^R) was donated by A.Scott. Cells were incubated in complete media overnight at 28°C and grown to 3-4 $x10^7$ cells/ml. They were then re-suspended in cold phosphate buffer saline (PBS) at $2x10^7$ cells/ml.

5.3.2 Probes/primers used for the analysis (Fig. 5.1).

Probe/primer 1 has an overhang of a hexadT, followed by a 5'biotin hexadN, and is complementary and anneals to the 3' end of the TS strand:

5'Biotin-gatagctttttttACACCATCTACTACATAATTAATTGATAGTAGTTTCCT3'

Annealing temperature (*ta*)=55°C

Probe/primer 2 has an overhang of a hexadT, followed by a 5'biotin hexadN, and is complementary and anneals to the 3'end of the NTS strand:

5'Biotin-gatagctttttttACGGACTTGATGCACGTGAAAAACCATTATTTAAA3'

ta=57°C

Probe/primer3 is a modified version of the probe 2, which is shortened by 6 dTs from the overhang and has no 5'biotin. probe 3 is Complementary and anneals to the 3' end of the NTS strand :

5'-gatagcACGGACTTGATGCACGTGAAAAACCATTATTTAAA3'

ta=57°C

Probe/primer 4 is a modified version of the probe 1 which is shortened by 6 dTs from the overhang and has no 5'biotin. probe 4 is complementary and anneals to the 3' end of the TS strand:

5'-gatagcACACCATCTACTACATAATTAATTGATAGTTTCCT3'

ta=55°C

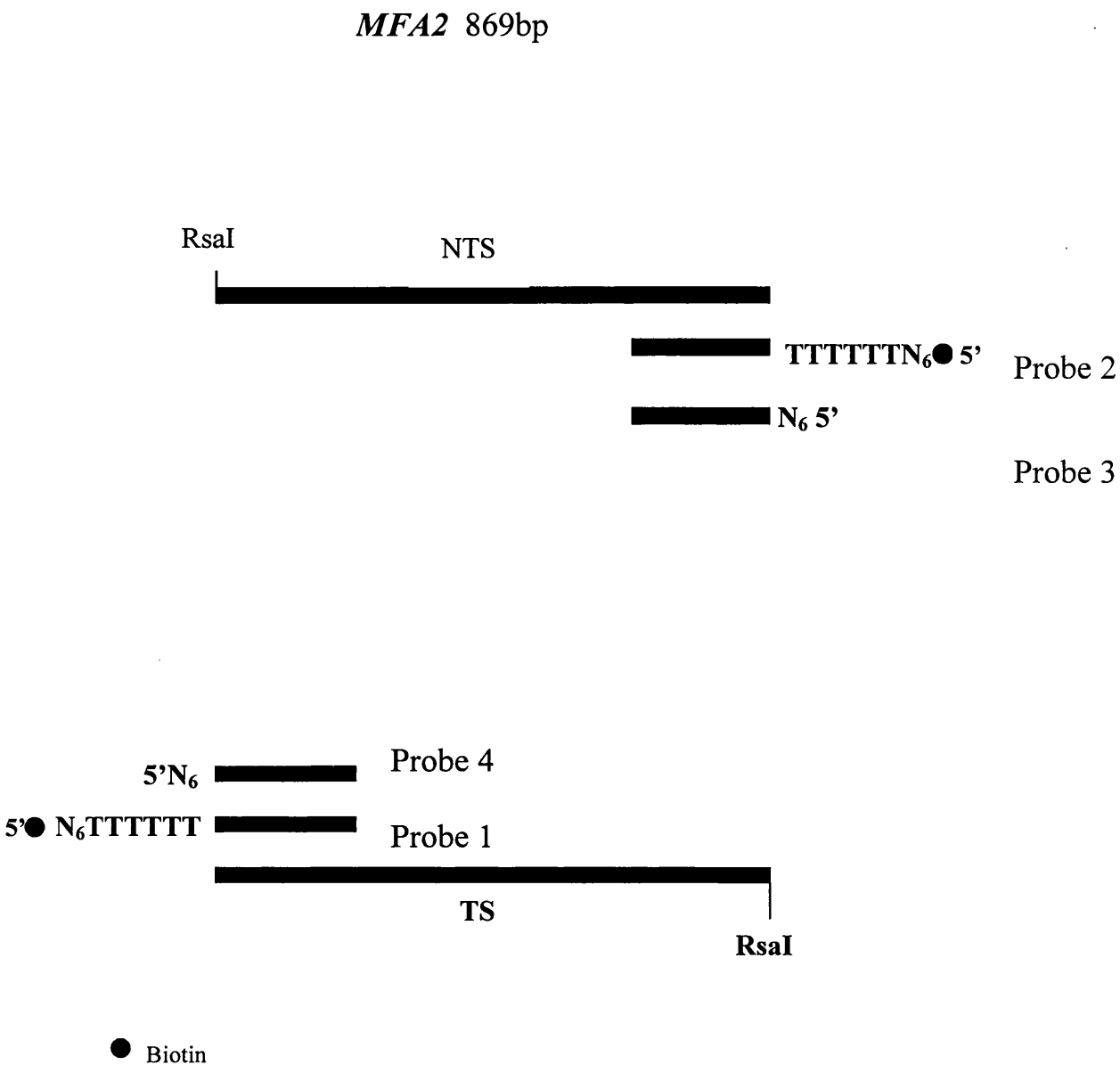


Figure 5.1 Primers/probes 1 and 2 are complementary and anneal to the TS and NTS of the *RsaI* fragment respectively. These primers have an overhang of a hexadT, followed by a 5' biotin hexadN. The primers 3 and 4 are modified versions of primers 2 and 1 respectively. The modification involves the removal of the biotinylated overhang and its replacement with only the haxadN (Teng *et al.*, 1997; Meniel *et al.*, 1999).

5.3.3 Fpg sensitive sites (FpgSS) and single strand break (SSB) induction

5.3.3.1 Chemicals

[R]-1[(10-Chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol or **RO 19-8022 (RO)** was a gift from Hoffmann- La Roche AG (Basel, Switzerland). **Methylene blue (MB)** was obtained from Sigma.

5.3.3.2 Treatment of the cells

Exponential phase cells from the overnight culture, were re-suspended in 20 ml ice cold PBS at $2x10^7$ cells/ml. 100µl of the RO chemical (1.2 mg/ml DMSO) was then added and the cell suspension was gently mixed and left on ice in the dark for 15 minutes. Subsequently the mixture was poured into a 25 ml petri-dish placed on ice and while being vigorously stirred by a magnetic stirrer, the cells were exposed to light from a 1000- Watt halogen lamp (Osram) at a 10 cm distance for 20 minutes. To maintain an optimal temperature of 4°C for treatment of the cells, an ordinary fan was operating in a minimum convenient distance from the work place and all of the operations were carried out in a 4°C cold room. At the end of treatment, the cell suspension was centrifuged at 3000 rpm and cells were re-suspended in 20 ml PBS. To prepare the cells for DNA extraction, 2 ml of EDTA were added. EDTA removes the magnesium ions that are necessary for preserving the overall structure of the cell envelope and it also inhibits the cellular enzymes that can degrade the DNA (Brown 1990). The cell suspension was kept on ice until DNA extraction. Special precautions were taken to minimise RO contamination and the RO treatment wastes contained in

plastic tubes were discarded into specifically assigned toxic waste bags before incineration.

5.3.3.3 DNA extraction

For each sample, 20 ml yeast cell suspension in ice cold PBS ($2x10^7$ cells/ml) into which 2ml EDTA had been added was centrifuged for 5 minutes at 3000rpm. To convert the cells to spheroplasts by removing the cell wall, cells were resuspended in 5ml Sorbitol solution into which, 500µl of Zymolyase plus 500µl of ß-Mercaptoethanol were added. The cells were gently re-suspended and incubated at 4°C overnight. After overnight incubation, 10µl of the cell suspension was viewed under a microscope to confirm that the spheroplasts were ready. The spheroplasts were then centrifuged at 3000rpm for 5 min, after which they were re-suspended in 5 ml of SEC buffer and were span down at 3000 rpm for 5 min. In order to lyse the spheroplasts, the pellet was re-suspended in 5 ml of a solution made with one volume lysis buffer plus one volume x1 PBS. 1.5ml RNAse A (1mg/ml in TE buffer)(Sigma) was added and the suspension was incubated for 2 hours at 37°C with occasional shaking. RNAse A degrades the RNA molecules into ribonucleotide subunits. After RNAse A treatment, 0.5ml of proteinase K (5mg/mlTE) (Biometra-Amresco) was added and the cells were incubated for 1 hour at 37°C with occasional shaking. The proteinase K breaks the polypeptides down into smaller subunits, which can be more easily removed by subsequent organic extractions. To purify the DNA from the proteins and cellular debris, one phenol chloroform extraction was carried out as

follows: an equal volume (7ml) of the mixed organic solvent phenol/chloroform:isoamyl (24:24:1) was added and the suspension was mixed by gentle inversion till it turned white. It was then centrifuged at 4000 rpm. The organic solvents precipitate proteins but leaves the nucleic acids in aqueous solution, resulting in formation of three layers upon centrifugation: a lower organic phase, a thin white layer at the interface containing the protein molecules, and the upper aqueous phase containing nucleic acids (Brown, 1990). The clear aqueous upper phase containing DNA was carefully removed by pipetting and transferred into a new tube. Another extraction was performed in the same way with an equal volume of chloroform /isoamyl alcohol (24:1). The clear supernatant was then transferred into a new tube. To precipitate DNA, 2 volumes of –20°C ethanol was added and the solution was incubated at –20°C overnight. The solution was centrifuged at 4000rpm and the pellet was re-suspended in 500µl of TE buffer in which the DNA re-dissolves. The DNA was precipitated by adding one volume of –20° isopropanol and mixing by gentle inversion. The floating DNA precipitate was collected with a pipette tip and was placed in a new Eppendorf tube. The short chain nucleic acid components including the ribonucleotides which remain in the solution, are lost during ethanol or isopropanol precipitation (Brown 1990). Finally the DNA pellet was left to dry at room temperature and was re-suspended in 500µl TE buffer. The DNA was stored at –20°C until used.

5.3.3.4 *In vitro* DNA treatment

In the dark, 2.5 µl of methylene blue solution (1mg/ml) was added to 300µl of distilled H_2O. This solution was added to 200µl of total yeast DNA (100µg/ml) extracted as previously described. The total volume of the MB treated DNA was pipetted into one well of a 24-well plate (Nunc Multidish)which was placed on ice and exposed to a 150 Watts Bulb (Claudfar) at a 20 cm distance. The DNA was washed by precipitation with ethanol at –20°C. Appropriate care was taken to minimise MB contamination.

5.3.3.5 Isolation of *MFA2* fragment by *RsaI* digestion

In order to isolate the *MFA2* gene, 200µl of a total DNA solution equal to approximately 60 µg DNA from cells, of each sample was restricted with 120 units of RsaI (Gibco BRL restriction enzyme). The restriction was carried out at 37°C for 1 hour in a total reaction volume of 300µl made up with H_2O and corresponding restriction buffer as described in the protocol supplied by the manufacturer. The enzyme was then removed by one phenol/chloroform and one chloroform extraction, as described earlier. To precipitate the restricted DNA 0.1 volume of 3 M NaCl (30µl) and one volume of iso-propanol (300µl) was added to the DNA solution which was then incubated at –70°C for 10 minutes. The DNA precipitate was pelleted by centrifugation and resuspended in 200 µl TE buffer. Fig. 5.2 and 5.3 demonstrate the gel electrophoresis of DNA before and after *RsaI* digestion.

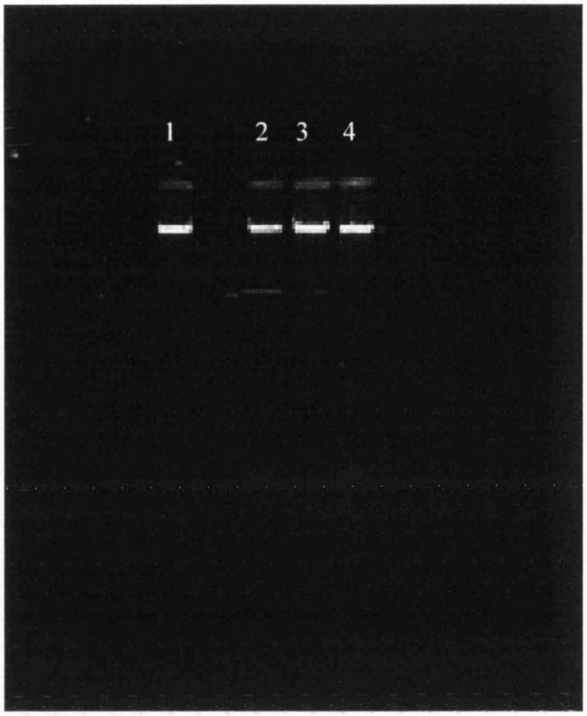


Figure 5.2 2μl of each DNA preparation (**1.** untreated DNA, **2.** MB-light treated DNA, **3.** DNA extracted from untreated cells, **4.** DNA extracted from RO-light treated cells) was loaded onto a non-denaturing 1% TAE agarose gel (containing 1μg/ml EtBr) and electrophoresed under 1 x TAE running buffer. Genomic DNA runs as a discrete high molecular weight band.

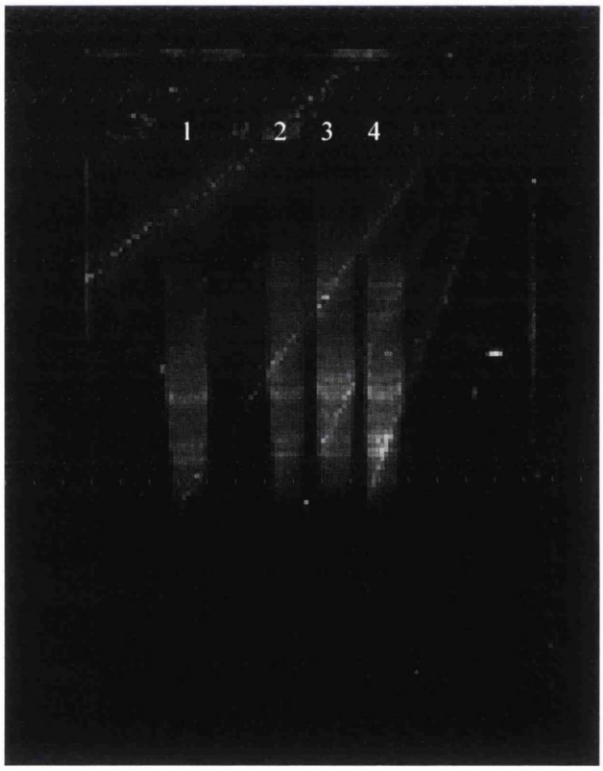


Figure 5.3 To confirm that DNA is successfully cut by restriction enzyme digestion, 4μl of restricted DNA (Restricted samples of 1,2,3,4 DNA as described in Fig. 5.1) was examined by electrophoresis under the same condition as described in Fig.5.1. Restricted DNA migrates as a smear. Distinct bands within the smear are representative of repeated sequences that exist within the yeast genome.

5.3.3.6 Fpg treatment

Each 200 µl *RsaI* digested DNA sample from the previous step was split into two 100µl portions. 0.5 µg Fpg/10µg DNA (i.e. 3µg Fpg) was added into one portion, which was then incubated at 37°C for one hour. The second aliquot was mock treated with the same volume of TE buffer. All samples were incubated for 1 hour at 37°C.

5.3.4 Isolating *MFA2* TS and NTS fragments

The process of isolation and end labelling of RsaI restriction fragments is illustrated graphically in Fig. 5.4. In order to isolate the TS fragment, 1 picomole (equal to 1µl) of the biotynylated probe1, which is complementary to and anneals to the TS, was added to each 100µl Fpg restricted or mock treated DNA sample from the previous step. The samples were denatured at 95°C for 5 minutes and then incubated at the annealing temperature of 55°C for 15 minutes to allow the probe to anneal to the 3' end of the TS of the *MFA2* fragment. 30µl of 5M NaCl was then added into the tube. To separate the TS of the *MFA2* fragments from the genomic DNA, 10µl of Dynabeads (10mg/ml) which had been previously washed twice with each of a x1PBS, 0.1%BSA solution and a binding and washing buffer (B&W), was added to each sample. The tubes were gently shaken for 15 min at room temperature to allow the Strepdavidin coated Dynabeads to bind the biotin molecule at the 5' end of the primer. The tubes were placed in the Dynal magnetic particle concentrator (MPC) and the beads containing the attached *MFA2* fragment were magnetically attracted to the wall of the tube. The supernatant containing the remaining genomic DNA was removed and stored for isolating the NTS fragment. The beads were washed with 60µl of B&W buffer for 5 minutes and the buffer was removed. The beads were washed

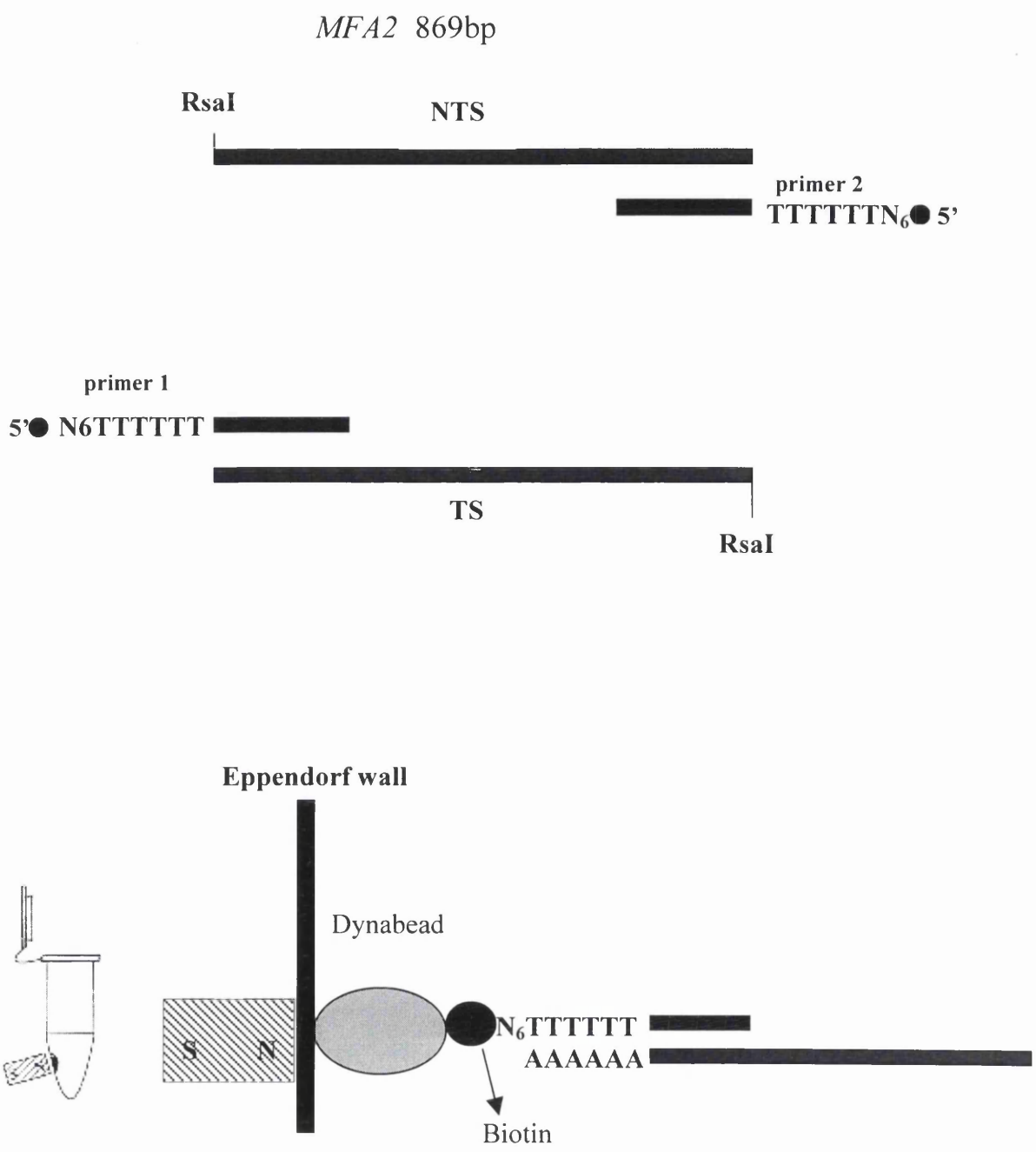


Figure 5. 4 The process of isolation and end labelling of *RsaI* restriction fragments containing *MFA2*. Top, the primers/probes 1 and 2 are complementary to 3' end of the TS and NTS respectively. At the end of each probe an overhang of a hexadT follows a 5'biotin hexadN. *RsaI* restricted DNA is denatured and annealed to either TS or NTS probe. Bottom, The annealed fragments from the previous stage are then isolated from genomic DNA using streptavidin-coated magnetic beads which binds the biotin. Subsequently, the beads with their bound sequences are captured in the tube by using a magnet placed at the side of the tube while the non-bound genomic DNA is washed away. Finally the *MFA2* fragments are end labelled with exactly 6 radio-labelled dATP using DNA polymerase. The labelled fragments are then eluted from the beads and separated on a DNA sequencing gel (*Adapted from Teng and Waters et al., 1997*).

twice more with 60µl of water and the water was removed. The beads bound to the TS *MFA2* fragment were resuspended in 6 µl water. The NTS fragment was isolated in the same way from the supernatant containing DNA other than the TS of *MFA2*, using probe 2 and an annealing temperature of 57°C.

5.3.5 End labelling the *MFA2* fragments

Annealed TS or NTS fragments were end labelled with exactly 6 [^{32}P] dATP molecules at the 3' end by using the six dTs at the 3' end of the probe 1 or probe 2 respectively as template (Fig. 5.4). The 6 dNs in the overhang that follow the six dTs, are not polymerised, as dATP is the sole nucleotide triphosphate available. These dNs are necessary to allow the complete polymerisation of the six dAs without hindrance from the biotin/streptavidin bead. 1µl Sequenase buffer, 0.7µl of 0.1 M DTT, 1µl (5µ Ci) of [^{32}P] dATP (Amersham 6000 Ci/mmol) and 2 µl of Sequenase (diluted 1:8 as instruction) were added to the beads associated with the TS or NTS of *MFA2*, suspended in 6 µl water from the previous step. The labelling reaction was allowed to complete for 10 minutes at room temperature. The labelling solution was removed and the labelled *MFA2* fragments attached to the beads were washed with 30µl of B&W buffer, and washed twice at room temperature with 30µl of TE. The labelled *MFA2* fragments were then eluted from the Dynabeads at r.t. by adding 3µl of formamide loading buffer (95% formamide, 20mM EDTA, 0.05% w/v bromophenol blue). The solution was stored at –20°C until gel electrophoresis.

3.3.6 Sequencing the *MFA2* fragment

A reference sequence ladder was required to determine the specific nucleotides in the sequence at which the oxidative base lesions occurred. A Sanger sequencing of the opposite strand adapted from Teng *et al.*, (1997), was employed. In this method the sequencing ladders are obtained by amplification of the *MFA2* fragment via PCR. Primers for the PCR reaction are designed so that one primer is biotinylated, allowing the PCR product for that strand to be separated from the complementary strand using Dynabeads, and the other non biotinylated primer is used to perform the sequencing reaction. In order for the sequencing ladder and the damage ladder to run in the same orientation, it is essential when analysing DNA damage in the TS to sequence NTS and vice versa. The PCR reactions are performed using primer 1 coupled with primer 3 for sequencing the TS and primer 2 coupled with primer 4 for sequencing the NTS. Primer 3 and 4 are modified versions of primers 2 and 1 respectively. The modification involves removal of the biotinylated overhang and replacement with a random hexanucleotide (Fig. 5.1). This allows the sequencing reaction products to run to the same position in the gel as the DNA damage ladder which is end labelled with 6 [^{32}P]dATP molecules.

In order to analyse the damage in the TS strand, the NTS of *MFA2* fragment was sequenced as follows; 20 µl of washed Dynal beads were added to 10 µl PCR product of the *MFA2* fragment made with primer 2 (biotinylated) and 4 (nonbiotinylated) (Fig. 5.1). The beads were allowed to bind to the biotin at the 5' end of the extended primer 2 of the double stranded PCR product by incubation at room temperature for

10 minutes with occasional shaking. The beads attached to the PCR product were collected by MPC and the supernatant was discarded. The beads were then re-suspended for 10 min at room temperature in 8µl of 0.1M NaOH to denature the double stranded DNA. The supernatant was discarded and the beads which were now carrying only the extended primer 2, were washed twice with 130 µl water and were then resuspended in 7 µl water. 2µl of reaction buffer and 1µl primer 4, were added and the tubes were incubated at 65°C in a water bath for two minutes. The tubes were allowed to cool gradually to a temperature of <35°C at room temperature for 15 minutes during which the primer 4 was annealed to its complementary sequence of the extended primer 2. Subsequently, 5.5 µl of the reaction mixture containing 2µl diluted Sequenase labelling Mix containing dNTPs, 0.5 µl of [^{35}S]dATP (10µCi/ml at > 1000 Ci /mmol), 2µl of Sequenase enzyme and 1µl 0.1M DTT were added to the 10µl solution containing the beads. The tubes were incubated at room temperature for 2 minutes. Four tubes labelled A,G,C,T, each containing 2.5 µl of one of the four termination mixture ddATP, ddGTP, ddCTP, ddTTP respectivly were prepared. 3.5µl of the reaction mixture was added to each termination tube and the tubes were incubated at 37°C for 5 minutes. To stop the reaction, 4µl of stop solution were added and the samples were heated at 80°C for 2 minutes before denaturing gel electrophoresis. The TS was sequences similarly using primer 1 coupled to primer 3.

3.3.7 Denaturing polyacrylamide gel electrophoresis

The damage ladders were loaded alongside the sequencing ladder onto the 6% (w/v) denaturing polyacrylamide gels. TBE was used as running buffer and the electrophoresis was carried out in a field of 40V/cm.

3.3.8 Detection of FpgSS and SSB

After electrophoresis, the sequencing gels were placed under a phosphor-imager screen. The gel was then scanned with a phosphor-imager scanner (Molecular Dynamics, Sunnyvale, CA, USA), which detects the 3' end labeled bands.

5.4 Results

The spectrum of in vivo (MB) or *in vivo* (RO) induced SSB and FpgSS in the TS and NTS strands of the *MFA2* gene of *S.cerevisiae* was investigated at nucleotide resolution as described in materials and methods.

The autoradiographs obtained are presented in Fig. 5.5, Fig. 5.6 and Fig. 5.7. T,A,C and G represent the reference sequence ladder. In each lane the top band represents the full-length single stranded fragment containing no damage. The strands resulted from single strand breaks or the strands incised by Fpg protein migrated at lower positions in the lane. There is a considerable increase in the intensity of the bands in the lanes containing DNA incubated with Fpg protein. The nature and the position of the bands in the damage ladders can be determined by comparing the nucleotide position in the sequence to the reference sequence ladder alongside. The amount of damage is reflected by band intensity and this can be determined by quantification of the fraction of activity in a given band. However, as the data in this section are from only one experiment and unfortunately there was no time to repeat the experiments, no detailed analysis of damage was undertaken.

Fig. 5.5 Induction of FpgSS in the *MFA2 RsaI* fragment for the transcribed strand. The lanes represent DNA from untreated cells (U) and DNA treated *in vitro* by MB-light (MB). DNA was incubated (+), or not incubated (-) with Fpg enzyme. The T,A,C,G lanes represent the sequencing ladders. The vertical bar represent the map of the *RsaI* fragment consisting of the promoter region i.e. nucleotides –225 to -43(white box). The transcribed region of *MFA2* i.e. nucleotides –43 to +285 (dark grey box)

with +1 representing the start of the coding sequence, and the nucleotides +285 to +435 (light grey box) which is the end of the *RsaI* fragment.

Fig. 5.6 Induction of FpgSS in the *MFA2 RsaI* fragment for the transcribed strand. The lanes represent DNA from untreated cells (U) and DNA extracted from the cells treated *in vivo* by RO-light (RO). DNA was incubated (+), or not incubated (-) with Fpg enzyme. The T,A,C,G lanes represent the sequencing ladders. The vertical bar represent the map of the *RsaI* fragment consisting of the promoter region i.e. nucleotides –225 to -43(white box). The transcribed region of *MFA2* i.e. nucleotides –43 to +285 (dark grey box) with +1 representing the start of the coding sequence. and the nucleotides +285 to +435 (light grey box) which is the end of the *RsaI* fragment.

Fig. 5.7 Induction of FpgSS in the *MFA2 RsaI* fragment for the non transcribed strand. The lanes represent DNA from untreated cells (U), DNA after *in vitro* treatment by MB-light (MB), and DNA extracted from the cells treated *in vivo* by RO-light (RO). DNA was incubated (+), or not incubated (-) with Fpg enzyme. The T,A,C,G lanes represent the sequencing ladders. The vertical bar represent the map of the *RsaI* fragment consisting of part of the promoter region from–43 upwards (white box). The transcribed region of *MFA2* i.e. nucleotides –43 to +285 (dark grey box) with +1 representing the start of the coding sequence (in the transcribed strand). and the nucleotides +285 to +435 (light grey box) which is the end of the *RsaI* fragment.

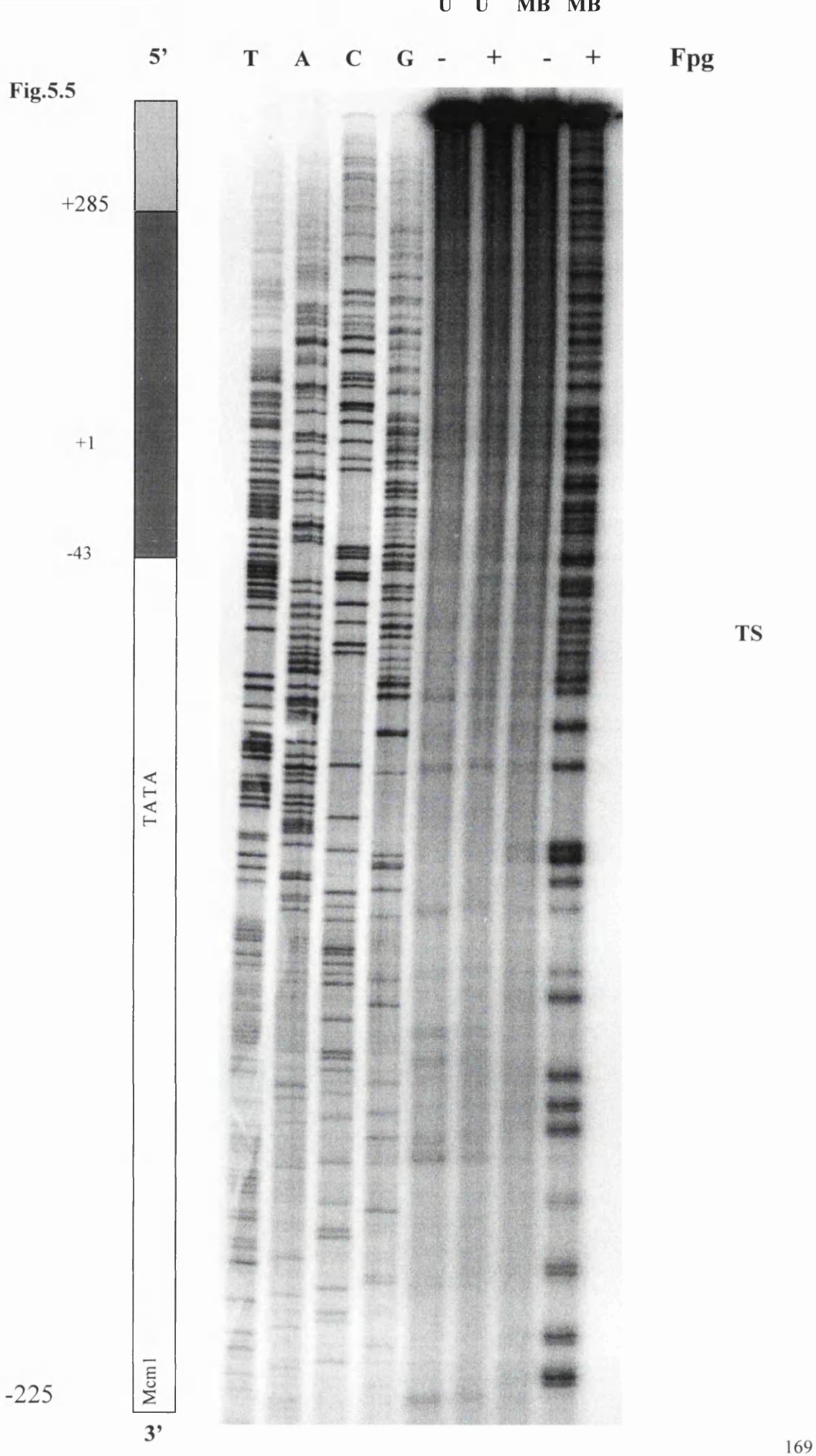


Fig.5.5

Fig 5.6

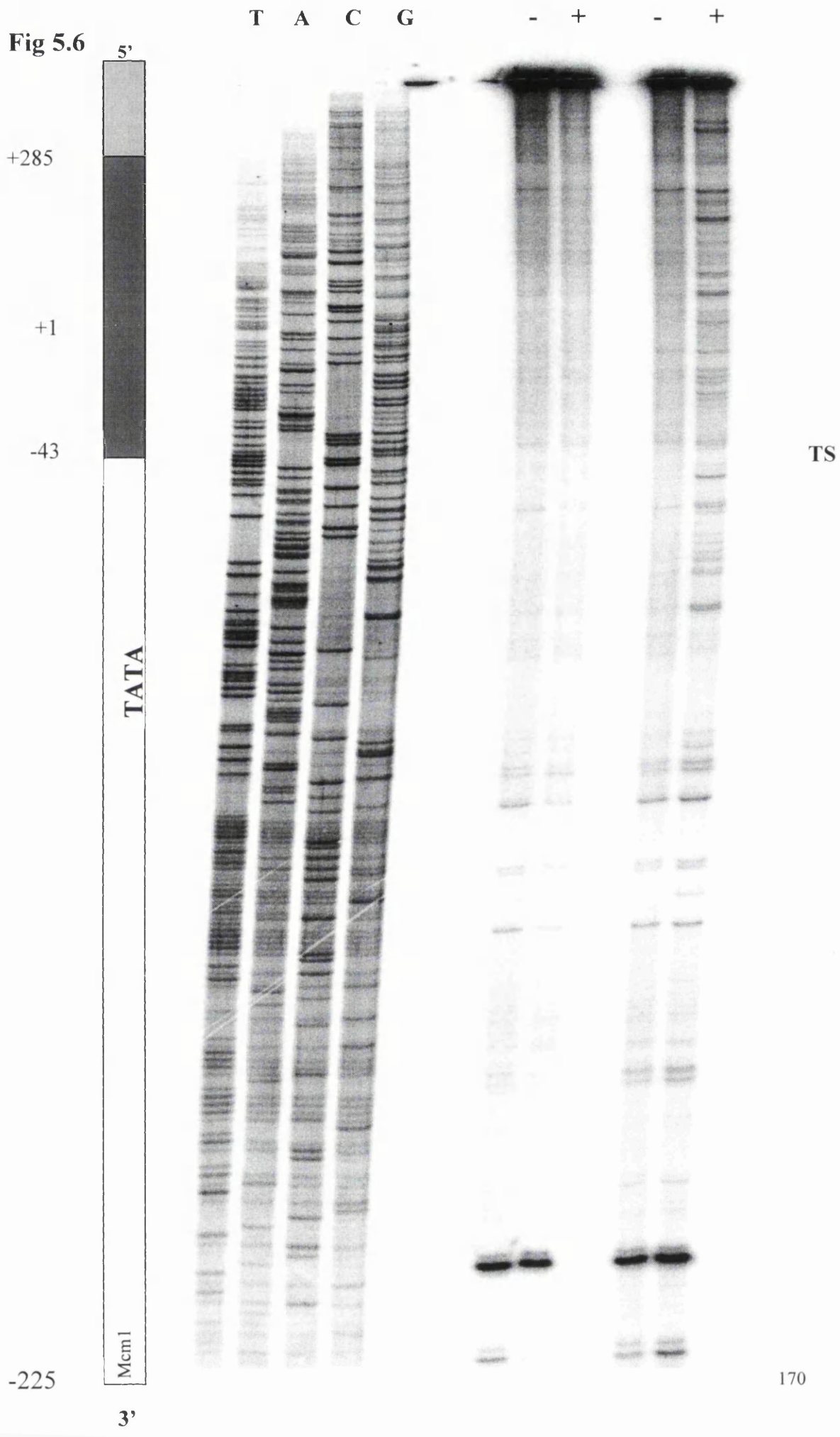
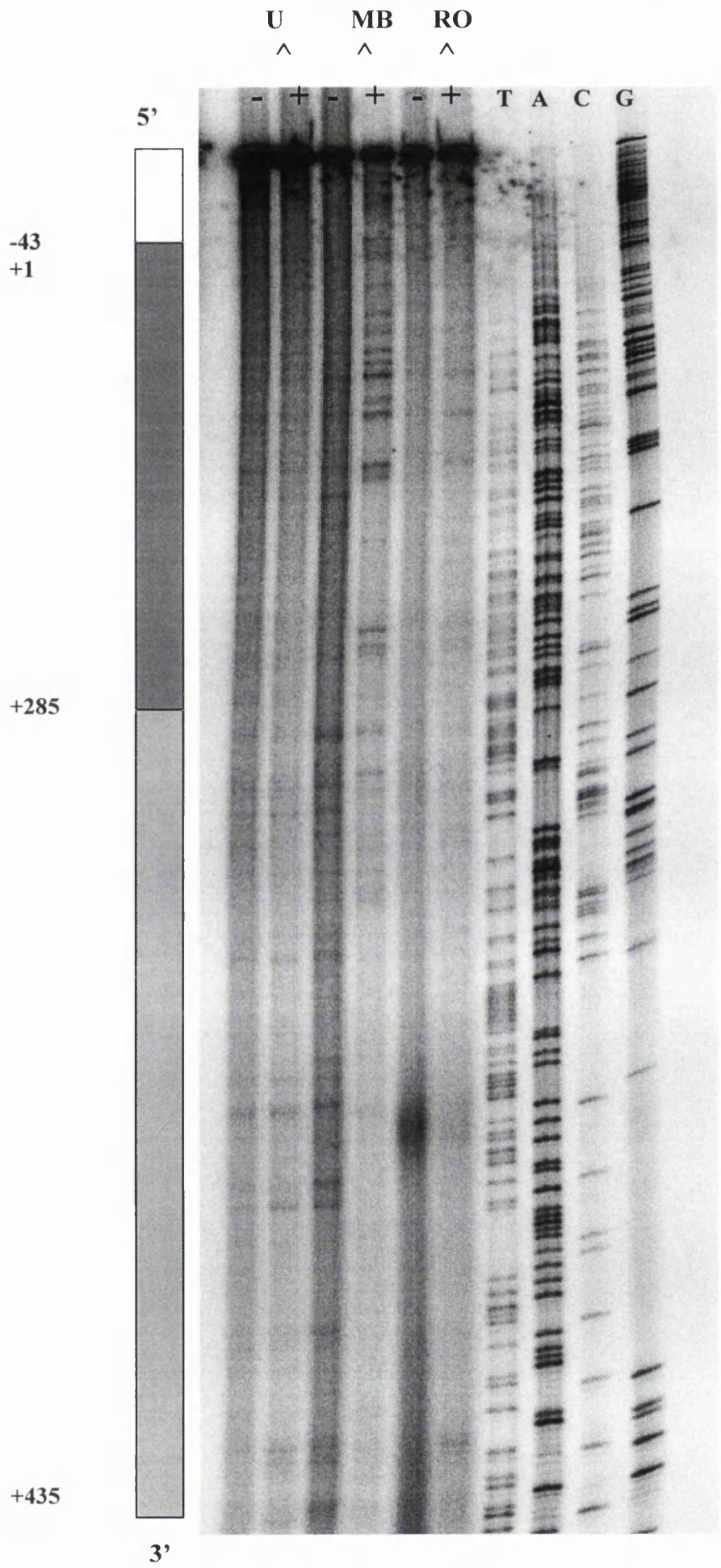


Fig 5.7



These results indicate that both the *in vitro* MB-light treatment of yeast DNA and the *in vivo* RO-light treatment of the yeast cells results in induction of FpgSS (bands in the lanes with Fpg protein) in DNA. However, the frequency of FpgSS induced by MB-light *in vitro* is higher than that induced by RO-light *in vivo*. The location of the hot spots also differs in two treatments. For both the *in vivo* and *in vitro* treated DNA, the bands corresponding to FpgSS occur more frequently at guanine rich regions, which span the transcribed region and the neighbouring promoter region close to the TATA box. From this region upstream, the frequency of FpgSS decreases. This corresponds to the region becoming less GC rich. For each treatment, there is a considerable variation in the intensity of the bands corresponding to FpgSS along the sequence which is possibly due to the variation in sequence context. From a visual comparison between the induction of damage in the NTS (Fig. 5.7) to that of TS (Fig. 5.5 and 5.6) of the *MFA2* fragment, it appears that the TS contains more damage sites than NTS.

Single strand breaks corresponding to the bands in the lanes without Fpg treatment are present in both treated and untreated DNA in same locations. Therefore these bands represent single strand breaks that may result during various stages of DNA extraction and purification. These bands are more frequent in the AT rich regions of the sequence.

5.5 Discussion

The method described by Meniel and Waters (1999) and in this thesis enables one to detect and define the spectrum of oxidative base damages at the level of nucleotide. This can be used as an assay to investigate the contribution of NER and BER to the repair of oxidative base lesions.

Here, this technique was used to investigate the spectrum of damage induced *in vitro* by MB light and *in vivo* by RO-light in the transcribed and nontranscribed strands of the *MFA2* gene of *S.cerevisiae*. In accord with previous findings (Meniel and Waters, 1999), the great majority of FpgSS induced by RO-light were at the same position as FpgSS induced by MB-light, which are at guanines. These data, together with the information previously obtained from HPLC analysis suggest that these damages are 8-oxoguanines. The frequency of FpgSS induced by MB-light *in vitro* is higher than that of induced by RO-light *in vivo*. This difference does not reflect the fragility of naked DNA versus chromosomally complex DNA, as it has been previously demonstrated that treatment of naked DNA with RO-light *in vitro* also gives less FpgSS and SSB compared to MB-light *in vitro* treatment. Therefore, the difference in the frequency of induction of FpgSS and SSB by RO-light and MB light is due to the nature of these chemicals and possibly a result of differences in the mechanism of formation of oxidative lesions. For example, MB light is a known intercalator (Ohuigin, 1987), whereas RO may not intercalate DNA.

All guanines, both after *in vivo* and *in vitro* treatment on naked DNA are hit but to varying extents. This suggests that the differences in induction are due to sequence context. Previous studies have demonstrated that most of the hot spots occur where

two or three guanines are present, regardless of the strand or the gene (Meniel and Waters, 1999).

It seems that induction of damage is higher in the TS (Fig. 5.5 and Fig. 5.6) than in NTS (Fig. 5.7) strand. It has been shown by Meniel and Waters (1999) that there are more damage sites/kb (SSB and FpgSS) in the TS of the *MFA2* compared to NTS. It has been suggested that this is due to the higher guanine content in the TS (181 guanine) compared to the NTS (114 guanine) of the RsaI fragment (Meniel and Waters, 1999).

The Fpg protein has an equal efficiency to cut at both fapyG and 8-oxoG, while the Ogg1 enzyme has a greater affinity for 8-oxoG and no Fapy activity of note. By using enzymes such as Ogg1, the 3' end labelling technique can be used to detect specific oxidative base lesions such as 8-oxoG. As it was mentioned earlier, very recent experiments using different doses of RO suggest that the high cytotoxicity of this chemical makes it very difficult to detect any repair in treated cells (Personal communications, Meniel, *et al.,* 2000). Nevertheless, by using a suitable oxidative DNA damage inducing chemical with an ability to induce specific oxidative base damages *in vivo,* with enough survival rate of the treated cells to detect DNA repair, it is possible to study the effects of transcription coupled repair or chromatin structure on the repair of induced oxidative base damages by analysing the damage and repair in the TS and NTS of a particular gene or in transcriptionally active or inactive genes (Meniel and Waters, 1999).

Chapter 6

General Discussion

Oxidative DNA damage has been implicated in the aetiologies of human pathologies such as cancer, ageing and many age-related diseases (Feig *et al.*, 1994; Wiseman *et al.* 1995; Beckman *et al.,* 1997). The DNA repair mechanisms operating on oxidative DNA lesions appear to be far more complex than previously considered. It has been widely assumed that the oxidative base modifications in DNA are repaired mainly by the BER pathway. However, as discussed in previous chapters, new evidence indicates that the role of NER in the removal of oxidative base lesions can not be underestimated. The mechanistic role of NER in this process is poorly understood and repair may depend on the nature or location of the oxidative base modification. Whether NER has only a back up role for BER, a crucial role on its own, or perhaps a synergistic role via interaction with BER enzymes, are subjects yet to be clarified. The aim of the research in this thesis was to investigate the contribution of NER to the repair of spontaneous or induced oxidative base modifications and to study the significance of this role compared to the role played by BER via the Ogg1 protein of *S.cerevisiae.*

To provide experimental tools for the study, a series of mutant strains were constructed to be deficient in NER, or both NER and BER. This was undertaken by disruption of the *RAD14* gene in the wild type and *ogg1* mutants respectively (chapter

2). In chapter 3 the frequency of spontaneous forward mutation to Can^R was examined in these mutants relative to the wild type. A summary of the result of these experiments is shown in Table 6.1 and Fig. 6.1. The raw data and statistical analysis are contained in Appendix III.b.11. Similar to the BER defective *ogg1* mutant, the NER defective *rad14* single mutant showed an elevated frequency of spontaneous mutations to Can^R relative to the wild type. These data indicate that NER is also involved in the repair of certain kinds of spontaneous base damages leading to spontaneous mutations. This result could imply that NER is acting on substrates that cannot be repaired by BER, as the lack of NER activity could not be compensated by BER in *rad14* single mutants. However, the double *ogg1rad14* mutant did not exhibit an additional increase in the frequency of spontaneous mutation relative to either single mutant, which would be expected if the two repair activities were operating on different lesions. Instead, the level of increase in spontaneous mutations relative to the wild type was similar for all three *ogg1*, *rad14,* and the *ogg1rad14* mutant strains. This finding suggests that there might be an interaction between the NER and BER repair activities for certain substrates. For instance a protein-DNA interaction between a NER protein and the lesion could recruit the BER repair factors to the damage site. Or, it is also possible that some direct protein-protein interactions between the NER and BER repair enzymes are necessary for the repair function to take place. This was further supported when it was shown that over-expression of the *OGG1* gene can decrease the spontaneous mutation frequencies down to the wild type level only in *ogg1* mutants but not in the NER defective *rad14* strain.

The response of the *ogg1rad14* mutants to over-expression of the *OGG1* gene can be interpreted in two ways; If we rely on the statistical analysis and accept that the slight

decrease in spontaneous mutation frequency in the *ogg1rad14* mutant is not significant when it is compared to the sharp decrease in *ogg1*+ pYSB10 strain, then this would support the necessity for an interaction between NER and BER repair proteins, by showing that Ogg1 was not able to suppress spontaneous mutations when NER was inactive. On the other hand, if the repeated pattern of a slight decrease in mutation frequency by over-expression of the *OGG1* gene in the *ogg1rad14* mutant is compared to the no decrease at all in the *rad14*+ pYSB10 mutant, it can be argued that this decrease could be biologically significant. This then would indicative that although for the repair of certain oxidative base lesions an interaction between NER and BER is necessary, some other lesions are substrates only for one of these mechanisms, as over-expression of the Ogg1 in *ogg1rad14* mutant resulted in neither a complete suppression like observed for *ogg1* + pYSB10, nor a complete lack of suppression as observed for *rad14* + pYSB10; but it demonstrated only a slight decrease in the frequency of spontaneous mutations. A summary of the results for those experiments is shown in Table 6.2 and Fig. 6.2. The raw data and statistical analysis are contained in Appendix III.b.12.

In support of an interpretation between two repair mechanisms, the first *in vitro* evidence of an interaction between a NER and BER protein has recently been demonstrated in two separate studies (Klungland *et al.,* 1999; Bessho, 1999). Thymine glycol, produced as a result of the oxidation of thymine, is a toxic oxidative base lesion that blocks DNA polymerases and introduces distortion in the double helix (Hayes *et al.,* 1988; Kung and Bolton, 1997; Purmal *et al.,* 1998). In humans, this lesion is excised by hNth1, the human counterpart of the bacterial Endonuclease III and the yeast Ntg1/Ntg2 protein. hNth1 also cleaves the DNA at the 3'side of the abasic site to generate a 3'terminal sugar phosphate residue that cannot be used as a

Total median for Exp.3.1-3.7			
Genotype	**Can^R/10^6**		
	Median	**LIQV**	**UIQV**
wild	0.23	0.177	0.39
ogg1	2.395	1.38	3.1825
rad14	2.25	1.97	2.59
ogg1rad14	1.72	1.29	2.53

Table 6.1 The frequency of spontaneous mutation to Can^R compared between the wild type, *ogg1* , *rad14* and *ogg1rad14* strains .The Can^R/10^6 values shown, are the total medians, lower and upper interquartile values (LIQV and UIQV respectively), obtained from the individual Can^R/10^6 values of 7 independent experiments (Exp. 3.1-3.7), each surveying a minimum of 5 independent colonies.

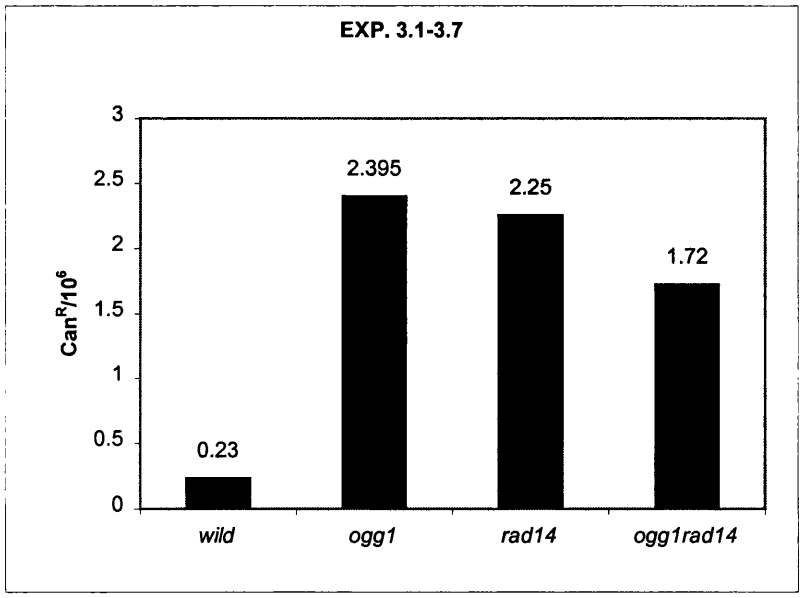


Fig. 6.1 . Graphical illustration of the total median values, represented in table 6.1

Total median for Exp. 3.5-3.7

can^R/10^6

	pYSB10-		pYSB10+		pYSB10-/pYSB10+
	Median	**(LIQV,UIQV)**	**Median**	**(LIQV, UIQV)**	
WILD	0.23	(0.185, 0.250)	0.25	(0.215, 0.275)	0.92
ogg1	1.83	(0.778, 2.605)	0.11	(0.060, 0.175)	17.43
rad14	2.35	(2.210, 2.488)	2.28	(2.005, 2.450)	1.03
ogg1rad14	1.96	(1.400, 2.605)	1.26	(0.900, 1.9)	1.56

Table 6.2 The effect of overexpression of the *OGG1* gene by plasmid pYSB10, on the frequency of spontaneous mutation to Can^R in the wild type, *ogg1* , *rad14* and *ogg1rad14* strains of *S.cerevisiae* .(The Can^R /10^6 values are the total median, lower and upper interquartile values (LIQV and UIQV respectively), obtained from the individual Can^R /10^6 values of 3 independent experiments (Exp.3.5-3.7) each surveying a minimum of 5 independent colonies.

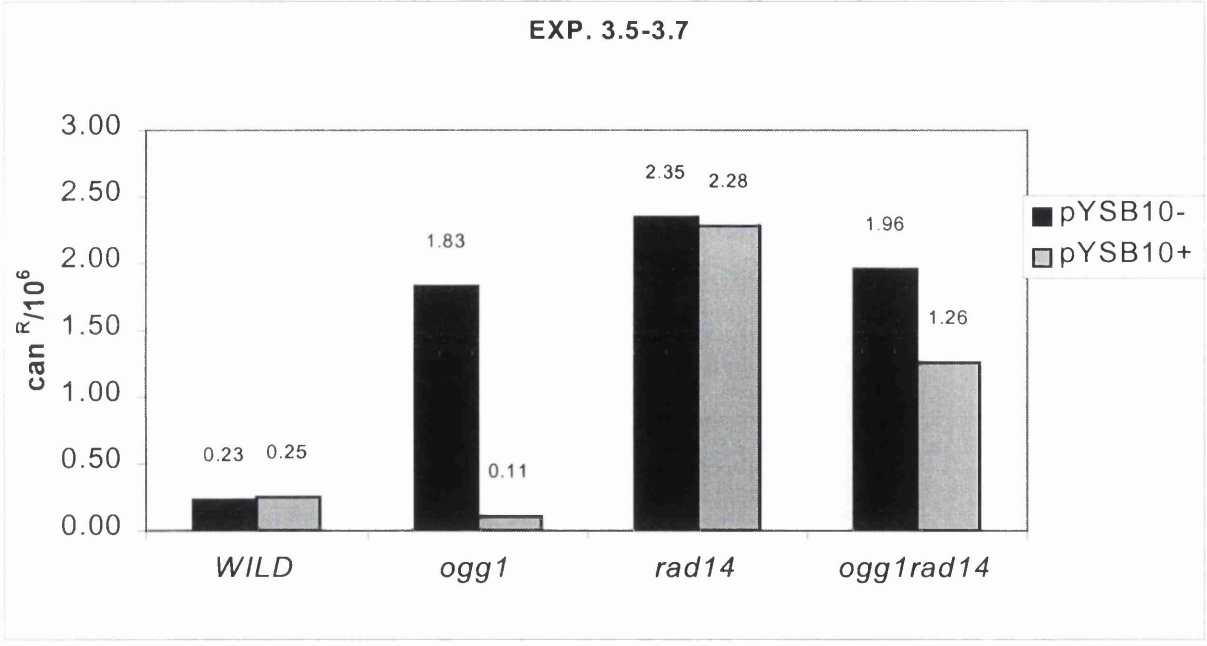


Fig. 6.2 Graphical illustration of the total median values represented in table 6.2

primer for subsequent DNA synthesis. The removal of this 3' sugar phosphate residue is achieved via cleavage of the phosphodiester bond on the 5' side of the abasic residue by the AP endonuclease HAP1 [also called APE] (Demple and Harrison, 1994). This is followed by filling in the one nucleotide gap by DNA polymerase ß (Sobol *et al.,* 1996) and DNA joining by XRCC1-DNA ligase III (Cappelli *et al.,* 1997). Klungland and co-workers (1999) have reconstituted the *in vitro* BER of a double stranded oligonucleotide containing a Tg residue opposite adenine using the purified human BER enzymes mentioned above. Inefficient activity of moderate levels of hNth1 in the reconstituted BER reaction suggested the requirement for a promoting factor. It had been previously shown that XPG patients with early onset of Cockayne syndrome, produce only truncated XPG protein (Vermeulen *et al.,* 1993; Nouspikel *et al.,* 1997), a structure specific endonuclease responsible for DNA strand cleavage on the 3' side of a lesion during NER (O'Donovan *et al.,* 1994, Cloud *et al.,*1995; Evans *et al.,* 1997). The cells from these XPG-CS patients in addition to a defect in NER, show a reduced ability to remove Tg from their DNA exposed to ionising radiation (Cooper *et al.,* 1997). This defect is more striking during transcription coupled repair but it is also observed in general transcription-independent DNA repair (Cooper *et al.,* 1997). This information led Klungland *et al* (1999) to examine the XPG protein as a candidate cofactor promoting the BER of thymine glycol. Interestingly, the excision of thymine glycol and dihydrouracil form DNA by hNth1, was found to be strongly stimulated by XPG protein in a reconstituted BER reaction. Subsequently, it was suggested that the stimulatory effect of XPG on the hNth1 activity stems from interaction of XPG with the DNA base lesion or with hNth1 or both. No evidence of a direct protein-protein interaction was observed by using co-immuno-precipitation and affinity immobilization assays.

However, using special protein columns, Bessho, (1999) has demonstrated that hNth1 binds specifically to XPG *in vitro*. Furthermore, using a band shift assay it was shown that XPG protein enhances the binding of hNTh1 to the DNA substrate containing Tg, but it does not bind to DNA containing the UV-induced 6-4 photoproduct in both the presence or absence of XPG, indicating that XPG does not stimulate a non-specific binding of hNth1 (Klungland *et al.,* 1999; Bessho, 1999). These data suggested that XPG protein is involved in the damage recognition step of the thymine glycol BER (Bessho, 1999).

The same scenario for NER-BER interaction may exist in *S.cerevisiae*, and it would explain the absence of a significant difference between the level of increase in spontaneous mutation frequencies relative to the wild type between *ogg1*, *rad14* and *ogg1rad14* mutants. In support of this, it has been shown that the stimulation of hNth1 by XPG is not unique to this human DNA glycosylase, as the homologous *Schizosaccharomyces pombe* enzyme Nth-Spo was also shown to be activated by XPG. This is probably achieved in vivo by the *S.pombe* homologue of XPG, Rad13 (Klungland *et al.*, 1999; Carr *et al.,* 1993).

It has been shown that *S.cerevisiae* possesses two functional homologues of *E.coli* Endonuclease III, These are the Ntg1 and Ntg2 proteins [also called Scr1 and Scr2 respectively], (You *et al.*, 1998). Using the same approach as Bessho, (1999) and Klungland (1999) it would be appropriate to investigate whether there are any protein –protein interactions between Ntg1/Ntg2 and XPG or its *S.cerevisiae* homologue, Rad2.

In a forward mutation assay, the nature of the mutagenic lesions is not known, and this can limit our ability to interpret the results. In chapter 4 a specific reversion mutation assay (Hampsey, 1991) was used to measure the levels of spontaneous GC to TA transversions, a mutation induced by 8-oxoG. This permitted me to specifically investigate the contribution of NER and BER to the repair of 8-oxoG. No increase in the frequency of spontaneous mutation was observed in the *rad14* mutant relative to the wild type. A summary of these data is shown in Table 6.3 and Fig. 6.3. The raw data and statistical analysis are shown in Appendix IV.b.5 There is a significant increase in spontaneous GC to TA mutation in the *ogg1* mutant relative to the wild type, suggesting that 8-oxoG at this site is removed mainly by BER, and either NER has no role in removing this lesion or its role is masked in the presence of BER. The additional increase in the GC to TA mutation frequency in *ogg1rad14* mutants compared to the single *ogg1* mutants supports the latter interpretation as summarised in Table 6.4 and Fig. 6.4. The raw data and statistical analysis are contained in Appendix IV.b.5. It would appear likely that under normal growth conditions, 8-oxoG is repaired mainly by BER without any need for NER activity, and that this mechanism on it's own is perfectly capable of maintaining a wild type level of 8-oxoG in the genome. However, when BER is inactive, accumulation of large amounts of 8-oxoG may induce the expression and the activity of the NER proteins to target 8-oxoG lesions; i.e. the contribution of NER to the removal of 8-oxoG is only significant under conditions where BER is inactive or the BER pathway is saturated due to the presence of large amounts of oxidative damage. This possibility can explain the additional increase in the frequency of spontaneous GC to TA mutation in the double *ogg1rad14* mutant relative to the single *ogg1* mutant, and the fact that there was no increase in the single *rad14* strains relative to the wild type.

Total median for Exp. 4.1-4.4

	$CYC^+/10^7$		
	Median	**LIQV**	**UIQV**
wild type	0.03	0.02	0.04
ogg1	0.155	0.09	0.48
rad14	0.025	0.02	0.05

Table 6.3 The total frequency of spontaneous CYC^+ reversion compared between the wild tpe, *ogg1and rad14* mutants. The $CYC^+/10^7$ values are the median, upper and lower interquartile vlalues (LIQV, and UIQV respectively) obtained from the individaul $CYC+/10^7$ values of 4 independent experiments (Exp. 4.1-4.4) each surveying a minimum of 5 independent colonies

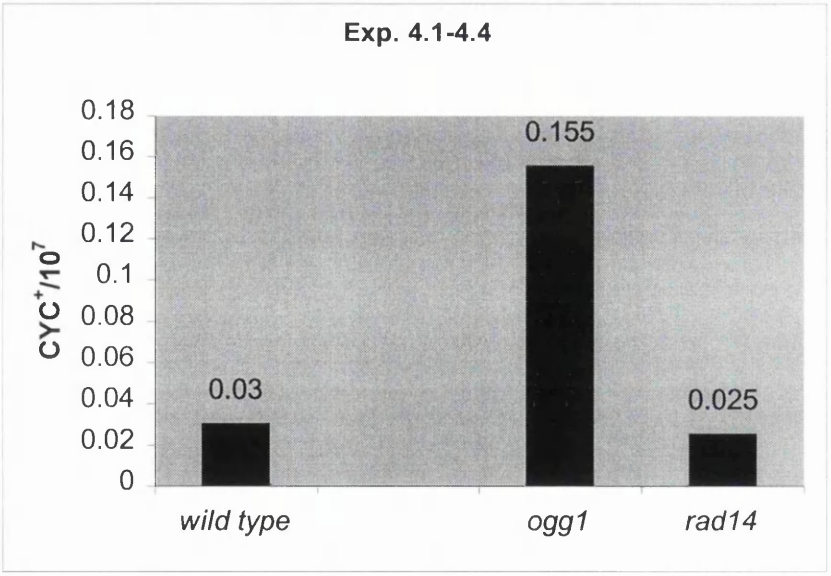


Fig 6.3. Graphic illustration of the total median values represented in table 6.3.

Total median of Exp. 4.1-4.4

	$CYC^+/10^7$		
	Median	**LIQV**	**UIQV**
ogg1	0.155	0.09	0.48
ogg1rad14	4.11	0.62	25.075

Table 6.4 The total frequency of spontaneous CYC^+ reversion compared between the the *ogg1* and *ogg1rad14* mutants. The CYC+/10^7 values are the median, upper and lower interquartile values (UIQV and LIQV respectively), obtained from the individual $CYC^+/10^7$ values of 4 independent experiments (Exp.4.1-4.4), each surveying a minimum of 8 independent colonies

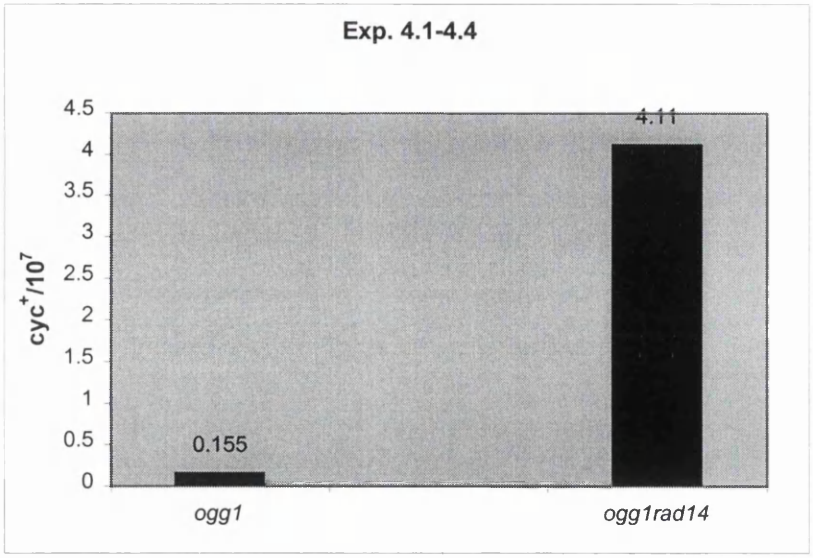


Fig 6.4. Graphic illustration of the total median values represented in table 6.4.

For example, it is possible that, when large amounts of 8-oxoG remain un-repaired, some electrochemical interactions between them or other bases cause distortion in the double helix and it is this distortion that is recognised by NER, and not the 8-oxoG *per se*. In support of this, it has been reported that 8-oxoG can be excised by human NER enzymes as part of a 25-30 base long oligonucleotide (Reardon *et al.*, 1997).

There is evidence indicating that 8-oxoG is highly susceptible to secondary oxidation, providing a likely target for endogenous oxidising agents such as peroxynitrate. Liquid chromatography/electrospray ionisation mass spectrometry (LC-ESI-MS) studies have demonstrated that oligonucleotides containing 8-oxoG can readily be oxidised by peroxynitrate *in vitro*, and the likely secondary oxidation product is oxaluric acid (Tretyakova *et al.*, 1999). It is possible that, when BER is inactive or BER proteins are saturated by large amounts of the induced damage, the accumulated 8-oxoG lesions give rise to a large number of secondary oxidation products that could also be helix distorting. These may then be substrates for NER.

The role of NER in the repair of 8-oxoG remains an open question; since despite our data and evidence that strongly support a role for NER in the removal of 8-oxoG, some other studies using reconstituted NER *in vitro* have been unable to detect any significant involvement of NER in the removal of 8-oxoG (Bohr *et al.*, 1998). However, if a combination of NER and BER proteins is required, these experiments would not have elucidated it. It would be useful to examine the spontaneous mutation frequencies in *ogg1*, *rad14* and *ogg1rad14* mutants of other Hampsey strains (described in detail in section 4.3.1) to study the contribution of NER and BER to the control of other specific base substitution events.

The results of the experiments in this thesis certainly suggest a role for NER in removal of oxidative base lesions. The results of spontaneous forward mutation assay for the detection of nonspecific spontaneous mutations indicate that certain oxidative base lesions can only be removed by interaction of two repair mechanisms, NER and BER, and for repair of those substrates, the absence of one can not be compensated by the other. Nevertheless, the nature of this interaction remains unclear. These experiments did not exclude that although for the repair of certain oxidative base damages an interaction between NER and BER is essential, some other oxidative base lesions can be substrate for only one mechanism. The results obtained from the GC to TA reversion mutation assay indicated that BER is the principal mechanism for the removal of 8-oxoG, but NER can also have a back up role for BER in removal of this important lesion.

In summary, in terms of repair, oxidative base lesions can be divided into 3 major groups: 1. Substrates that can be repaired by both NER or BER mechanisms alone, although the efficiency of the two pathways for this repair may differ for a particular lesion., 2. Substrates whose repair depends on an interaction between NER and BER repair mechanisms and they can not be repaired by NER and BER alone., 3. Substrates that can be repaired by only one of these mechanisms and are not substrate for the other.

However, this can not be a clear-cut division and apart from the type of the lesion, there are other factors in determining which repair mechanism or mechanisms have to be involved in removing a particular lesion. The Lys^+ reversion system employed by Scott *et al.,* (1999) suggested that NER may not have a role in the repair of the oxidative base lesions in the genes transcribed by RNA polymerase III such as tRNA

genes, as opposed to those transcribed by RNA polymerase II. It is also suggested that the location of the lesion in the genome is a factor that effects the contribution of a repair mechanism to the repair of a particular type of oxidative base lesion (Scott *et al.*, 1999). For example, for a certain oxidative base lesion that is normally a substrate for NER, the accessibility of NER complex can be hampered by assembly of certain regulatory factors in certain parts of the genome. This further complicates the research on contribution of NER and BER repair mechanisms to the repair of oxidative base damage. In the two assays used in my research , we were looking at the contribution of the two repair mechanisms on the frequency of spontaneous mutations in two completely different loci. Therefore our results may also reflect a contribution or lack of contribution of NER to the repair of certain oxidative base lesions depending on the location of the lesion in the genome. What ever the case, the story so far implicates that for the repair of oxidative base lesions and maintaining a normal level of spontaneous mutations NER as well as BER is essential to be functional.

In Chapter 5, the application of a technique recently developed to examine oxidative base damage at the level of nucleotide was described. This powerful approach can identify and quantify many specific oxidative base modifications at nucleotide resolution using appropriate repair enzymes to cut at their substrates.

Another method used to identify oxidative base damage at nucleotide level is ligase-mediated PCR (LMPCR). This relies on repair enzymes or chemical treatments that can produce ligatable ends after cutting DNA at damaged sites. These ends are ligated to a linker. Subsequently the cut fragment is amplified by PCR using a gene specific primer and a linker primer (Pfeifer *et al.,* 1991; Pfeifer *et al.*, 1992; Rodriguez *et al.*,

1995)). This method is technically more demanding and is advantageous when a limited amount of DNA is available such as in experiments with mammalian cells. However, it has the disadvantage that many repair enzymes do not produce ligatable ends at cut sites and these have to be generated by further processing. Furthermore, differential PCR, depending on fragment size could distort the perceived frequency of lesions. Thus, for research in yeast our 3' end-labelling technique is ideal.

The 3' end-labelling technique can be employed to investigate the repair of oxidative base damage at nucleotide resolution in repair competent cells and those defective in NER and/or BER. This approach can provide valuable data to elucidate the contribution of NER and BER to the repair of oxidative base lesions. Furthermore, it allows us to study the effects of factors such as location of the lesion in the genome, its frequency, and its flanking sequences in relation to transcriptional status and chromatin structure. By combining this approach with a reporter sequence fused to the yeast sequence under study, it is possible to relate damage and repair data to mutation at nucleotide resolution. Thus, many of the questions remaining with respect to the specific role of BER and NER in processing oxidative damage should be answered.

Appendix I- Recipes

Growth Media

LB medium

10g Bacto-tryptone; 5g Bacto-yeast extract; 5g NaCl. Made up to 1000ml with H_2O. LB plates contained in addition 15g agar.

YNBD minimal medium

0.7% Yeast nitrogen base w/o amino acids and 2% Glucose. YNBD plates contained in addition 2%Bacto-Agar.

YPD medium

1% Yeast extract; 0.5% Bacto-peptone and 2% Glucose. YPD plates contained in addition 2% Bacto-Agar.

YPG medium

1% yeast extract; 0.5% Bacto-peptone and 3% Glycerol; YPG plates contained in addition 2g Bacto-Agar per 100ml.

Buffers and Solutions

AE buffer

50mM Na acetate pH 5.3, 10mM EDTA.

Betamercaptoethanol

Add 1ml betamercaptoethanol to 49ml H_2O_2

100 X Denhardt's solution

1 g Polyvinylpyrrolidone (MW 40,000); 1 g Bovine Serum Albumin; 1g Ficoll 400; add distilled H_2O to make 50 mls and sterile filter. Store at 4°C or aliquot and store frozen.

2X Dynal beads washing and binding buffer

2 M NaCl, 10mM Tris-HCl (pH 8.0), 1mM EDTA.

1M DTT (dithiothreitol)

Dissolve 6.18 g of DTT in 40 ml of 10mM sodium acetate (pH 5.2). Filter sterile and aliquot. Store at –20°C.

EDTA (ethylenediaminetetra acetate) 0.5 M

Dissolve 186.1g disodium ethylenediaminetetra-acetate.$2H_2O$ (mol.wt. 372) in 800 ml sterile double-distilled H_2O by adjusting to pH 8.0 with 20.0 g of NaOH pellets. Make up to 1 liter with sterile, double-distilled water, aliquot and autoclave.

Lithium Acetate (LiAc) x10

1 M lithium Acetate.$2H_2O$; pH 7.5; filter sterile

Lysis Buffer

4M Urea; 0.2M NaCl; 100mM Tris-HCl (pH 8.0); 10mM EDTA; 0.5% (w\v) n-Lauroyl Sarcosine. Do not autoclave.

PBS (Phosphate Buffer Saline)

Dissolve 8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24g KH_2PO_4 in 800 ml of sterile double distilled water. Adjust to pH 7.4 with HCl and adjust volume to 1 liter . Aliquot and autoclave. Store at RT.

PEG (Polyethylene glycol) 50% (Average molecualr weight = 7000-9000

5 g PEG in 10 ml sterile H_2O; pH is not adjusted

Phenol chloroform isoamylalcohol (25:24:1)

Dissolve 250g phenol in 250 ml chloroform isoamylalcohol (24:1). Equilibrate the mixture by extracting several times with 0.1 M Tris-HCl (pH 7.6) Store solution under an equal volume of 0.1M Tris-HCl (pH 7.6) in a dark bottle at 4°C.

SDS (Sodium dodecyl/lauryl sulfate) 10% w/v

Dissolve 100 g of electrophoresis grade SDS in 900 ml H_2O by heating to 65°C; Adjust pH to 7.2 with conc. HCl; and aliquot. No need to sterilize, store at RT.

SEC buffer

1.0 M Sorbitol; 100 mM EDTA; 10mM Citrate Phosphate buffer (pH 5.8).

Sorbitol solution

0.9M sorbitol, 0.1M Tris-HCl (pH 8.0), 0.1M EDTA (do not autoclave).

20 X SSC buffer

3 M NaCl (175.35 g); 0.3 M Sodium Citrate Dihydrate (88.23 g); add distilled water to make 1 liter.

20 X SSPE

3 M NaCl (175.3 g); 0.2 M NaH_2PO_4-H_2O (27.6 g); 0.02 M EDTA -Na_2 (40mls of 0.5M stock solution), add 800 mls of distilled water, Adjust pH to 7.4 with NaOH, Add distilled water to make 1 liter.

Pre-Hybridization/Hybridization buffer

0.5M $NaHPO_4$ pH 6.5; 0.5 M EDTA; 20% SSC; 100% Denhardts; 10 X SDS; distilled H_2O.

TE X 10

100 mM Tris-HCl pH 7.5 ; 10mM EDTA Na_2; pH 7.5; filter sterile.

Tris-HCl pH 7.5 (1M)

Dissolve 121.1 g of Tris base in 800 ml sterile double-distilled water, allow solution to cool, and adjust to pH 7.5 with concentrated HCl (total vol. of 70ml). Adjust vol. to 1 liter with sterile, double distilled H_20.

Electrophoresis Buffers

10 X MOPS buffer

0.4 M Morpholinopropanesulfonic acid (83.7 g) ; 0.1M Sodium Acetate-$3H_2O$ (13.6 g); 10 mM EDTA-Na_2-$2H_2O$ (1.8 g), Adjust pH to 7.2 with NaOH, Add distilled water to make 1 liter.

Nondenaturing TAE (Tris –Acetate EDTA) electrophoresis running buffer

Dissolve 4.84 g Tris base (40mM), 0.372 g EDTA.Na_2.$2H_2O$ (1mM) in 800 ml distilled water and adjust to pH 8.0 with glacial acetic acid. Then made up to 1 liter with distilled water.

1 X TBE (Tris-Borate) buffer

10.8 g Tris base (89 mM), 5.50 g boric acid (89 mM), 0.93 g EDTA. Na_2.$2H_2O$ (2.5 mM); made up to 1 liter with distilled water. Note Boric acid adjusts pH to 8.3 at 25°C.

Loading buffers

Denaturing loading buffer for DNA

50 mM NaOH; 1mM EDTA; 2.5% Ficol; 0.025% Bromocresol Green

Denaturing loading buffer for RNA

720 µl Formamide; 160 µl of 10X MOPS; 260 µl 37% Formaldehyde; 180µl distilled H_2O; 80µl Bromophenol blue (stored at –20°C).

Non denaturing loading buffer for DNA

10% Ficol; 0.5% SDS; 0.06% Bromophenol Blue; Made up in 1x TAE.

Electrophoresis Gels

1% Agarose gel

3g Agarose in 300 ml 1x TAE buffer; 3µl ethidium bromide

6% nondenaturing polyacrylamide gel (used for PCR products)

The non-denaturing acrylamide solution (30% liquid acrylamide: bisacrylamide [37:5:1] NBL Gene Science) was made with 1/10 volume of 10X TBE buffer and distilled water to obtain the required volume. 450µl TEMED followed by 2.4mls ammonium sulphate was added to each 200 ml of this solution. *Bis acrylamide (Bis) is a cross-linking gent that causes the polymerisation of the monomers of acrylamide, forming a three dimensional matrix. The polymerisation is initiated by (APS) and the reaction is catalyzed by TEMED. The acrylamide/Bis ratio defines the eventual pore size, density, elasticity and mechanical strength of the gel.*

6% denaturing polyacrylamide gel (sequencing)

50 ml 6% polyacrylamide solution (acrylamide –Bis 19:1, (5.7% acrylamide, 0.3 %,Bis, 7 M ura, 0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA) (Amresco). 400µl APS 10%(Ammonium persulphate), 20µl Temed (N,N,N',N'-Tetramethylethylenediamine) .

1.5% RNA electrophoresis Gel

Dissolve 1g Agarose in 83 ml distilled H_2O and heat to boiling. Cool while stirring to 70°C. Add 10 ml of 10X MOPS buffer. While in the fume hood, add 5.4 ml of 37% w/v formaldehyde. Mix thoroughly and pour the gel.

Staining solutions

Silver staining solutions

First wash: 200 ml of distilled water. **Staining (for 10 min)** : 0.1% silver nitrate (Sigma-Aldrich), **After staining wash (2X)**: 200 ml distilled water. **Developing solution:** 300 ml of 1.5% NaOH, 0.15% formaldehyde (continue developing until the bands appear).

Ethidium Bromide staining solution

35µl Ethidium bromide (SIGMA [1mg/ml] in 1 liter distilled water.

Appendix II (Data for Chapter 2)

A II.1 UV sensitivity test for parental strains

YMH4 (Wild type)

UV Dose (J/m^2)	0			20			30			40		
dilution	x10^{-4}			x10^{-4}			x10^{-4}			x10^{-4}		
	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.
Plate	1	2		1	2		1	2		1	2	
Exp. 2.1	140	127	**100**	116	118	**88**	76	67	**53.6**	31	33	**24.0**
Exp. 2.2	165	130	**100**	124	140	**89**	82	75	**53.2**	35	42	**26.1**
Exp. 2.3	171	135	**100**	140	135	**90**	85	85	**55.6**	52	34	**28.1**
Mean			**100**			**89**			**54.1**			**26.1**

YOG4 (*ogg1*)

UV Dose (J/m^2)	0			20			30			40		
dilution	x10^{-4}			x10^{-4}			x10^{-4}			x10^{-4}		
	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv..
Plate	1	2		1	2		1	2		1	2	
Exp. 2.1	154	174	**100**	141	112	**77**	95	73	**51.2**	55	35	**27.4**
Exp. 2.2	173	180	**100**	164	176	**96**	82	75	**44.5**	43	38	**22.9**
Exp. 2.3	165	150	**100**	163	121	**90**	93	81	**55.2**	58	61	**37.8**
Mean			**100**			**88**			**50.3**			**29.4**

A II.2 UV sensitivity test for parental strains

FF18733 (Wild type)

0			20			30			40		
x10^{-4}			x10^{-4}			x10^{-4}			x10^{-4}		
col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.
1	2		1	2		1	2		1	2	
100	109	**100.0**	50	61	**53.1**	19	25	**21.1**	14	20	**16.3**
150	177	**100.0**	86	97	**56.0**	56	58	**34.9**	15	35	**15.3**
135	121	**100.0**	67	59	**49.2**	34	45	**30.9**	26	30	**21.9**
		100.0			**52.8**			**28.9**			**17.8**

CD138 (*ogg1*)

0			20			30			40		
x10^{-4}			x10^{-4}			x10^{-4}			x10^{-4}		
col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.
1	2		1	2		1	2		1	2	
83	55	**100**	33	28	**44.203**	24	29	**38.4**	13	19	**23.2**
109	120	**100**	55	72	**55.459**	53	69	**53.3**	35	40	**32.8**
104	123	**100**	40	61	**44.493**	30	34	**28.2**	25	36	**26.9**
		100			**48.052**			**40.0**			**27.6**

A II.3 UV sensitivity test for constructed YMH4 *derivative rad14* mutants

ADS304(*rad14)*

UV Dose (J/m^2)	0			5			10			15			20		
dilution	X10^0			X10^{-3}			X10^0			X10^0			X10^0		
	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.
Plate	1	2		1	2		1	2		1	2		1	2	
Exp. 2.1	132	119	**100.00**	50	30	**3.19**	211	216	**0.02**	17	12	**0.00**	5	6	**0.00**
Exp. 2.2	143	151	**100.00**	70	110	**6.12**	311	298	**0.02**	10	5	**0.00**	3	2	**0.00**
Exp. 2.3	150	171	**100.00**	100	130	**7.17**	250	231	**0.01**	12	12	**0.00**	8	9	**0.00**
Mean			**100.00**			**5.49**			**0.02**			**0.00**			**0.00**

ADS314 (*ogg1, rad14)*

UV Dose (J/m^2)	0			5			10			15			20		
dilution	X10^{-4}			X10^{-3}			X10^0			X10^0			X10^0		
	col. count		%surv.	col. count		%surv.	col. count			col. count		%surv.	col. count		%surv.
Plate	1	2		1	2		1	2		1	2		1	2	
Exp. 2.1	141	119	**100.00**	21	22	**1.65**	121	122	**0.01**	5	9	**0.00**	1	2	**0.00**
Exp. 2.2	156	130	**100.00**	50	71	**4.23**	114	100	**0.01**	8	12	**0.00**	0	3	**0.00**
Exp. 2.3	160	142	**100.00**	65	30	**3.15**	120	118	**0.01**	6	13	**0.00**	1	2	**0.00**
Mean			**100.00**			**3.01**			**0.01**			**0.00**			**0.00**

ADS110 (*ogg1, rad14*)

UV Dose (J/m^2)	0J			2J			4J			6J			
dilution	X10-4			X10-4			X10-2			X10-1			
	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.	col.
Plate	1	2		1	2		1	2		1	2		1
Exp. 2.1	121	140	100.00	27	23	19.16	13	20	0.13	21	42	0.02	38
Exp. 2.2	150	141	100.00	35	40	25.77	15	21	0.12	40	45	0.03	25
Exp. 2.3	136	149	100.00	41	37	27.37	21	19	0.14	34	30	0.02	21
Mean			**100.00**			**24.10**			**0.13**			**0.03**	

ADS110 (*ogg1, rad14*)

UV Dose (J/m^2)	0			2			4			6			
dilution	$X10^{-4}$			$X10^{-4}$			$X10^{-2}$			$X10^{-1}$			
	col. count		%surv.	col. count		%surv.	col. count		%surv.	col. count		%surv.	col
Plate	1	2		1	2		1	2		1	2		1
Exp. 2.1	88	96	100.00	17	25	22.83	80	96	0.96	70	60	0.07	30
Exp. 2.2	102	100	100.00	40	30	34.65	100	94	0.96	82	70	0.08	21
Exp. 2.3	123	145	100.00	35	36	26.49	103	98	0.75	50	67	0.04	41
Mean			**100.00**			**27.99**			**0.89**			**0.06**	

Appendix III (Data for Chapter 3)

Appendix III.a (Data and basic statistics)

A III.a.1

Exp. 3.1

Frequencies of Spontaneous Can^S to Can^R mutation

	YNBD plates (colonies x10^{-4})/100μl			YNBD+CAN plates colonies/400μl			$can^+/10^6$	$can^+/10^6$				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEV	Median	LIQV	UIQV
Wild type												
1	315	334	324.50	5	6	5.50	0.42	0.23	0.11	**0.21**	**0.17**	**0.21**
2	325	328	326.50	4	0	2.00	0.15					
3	270	331	300.50	2	2	2.00	0.17					
4	294	303	298.50	4	1	2.50	0.21					
5	273	310	291.50	2	3	2.50	0.21					
ogg1												
1	407	324	365.50	15	25	20.00	1.37	1.66	0.69	**1.39**	**1.36**	**1.41**
2	372	329	350.50	46	40	43.00	3.07					
3	345	312	328.50	16	19	17.50	1.33					
4	341	378	359.50	21	18	19.50	1.36					
5	402	433	417.50	20	27	23.50	1.41					
6	491	452	471.50	30	23	26.50	1.41					
rad14												
1	500	500	500.00	37	28	32.50	1.63	1.79	0.89	**1.51**	**1.26**	**2.09**
2	360	380	370.00	55	44	49.50	3.34					
3	790	590	690.00	37	30	33.50	1.21					
4	600	870	735.00	60	72	66.00	2.24					
5	670	600	635.00	28	43	35.50	1.40					
6	600	740	670.00	23	25	24.00	0.90					
ogg1rad14												
1	480	540	510.00	50	84	67.00	3.28	1.62	1.06	**1.31**	**0.92**	**2.24**
2	550	430	490.00	15	25	20.00	1.02					
3	620	510	565.00	41	31	36.00	1.59					
4	570	730	650.00	17	29	23.00	0.88					
5	560	560	560.00	9	14	11.50	0.51					
6	550	540	545.00	50	57	53.50	2.45					

A III.a.2

Exp. 3.2

Frequencies of Spontaneous Can^S to Can^R mutation

	YNBD plates (colonies x10^{-4})/100μl			YNBD+CAN plates colonies/400μl			$can^+/10^6$	$can^+/10^6$				
							$can^+/10^6$	Mean	STDEV	Median	LIQV	UIQV
plate	1	2	(1+2)/2	1	2	(1+2)/2						
Wild type												
1	103	81	92.00	2	4	3.00	0.82	0.56	0.25	**0.59**	**0.48**	**0.72**
2	302	295	298.50	3	13	8.00	0.67					
3	304	271	287.50	7	10	8.50	0.74					
4	200	200	200.00	3	5	4.00	0.50					
5	300	330	315.00	5	7	6.00	0.48					
6	283	277	280.00	2	1	1.50	0.13					
ogg1												
1	230	249	239.50	56	44	50.00	5.22	3.75	1.05	**3.28**	**3.22**	**4.42**
2	242	278	260.00	36	56	46.00	4.42					
3	283	221	252.00	28	37	32.50	3.22					
4	277	302	289.50	32	44	38.00	3.28					
5	241	390	315.50	35	31	33.00	2.61					
rad14												
1	410	680	545.00	54	44	49.00	2.25	2.88	0.93	**2.73**	**2.33**	**3.69**
2	360	580	470.00	56	52	54.00	2.87					
3	820	560	690.00	53	38	45.50	1.65					
4	450	350	400.00	32	51	41.50	2.59					
5	400	490	445.00	66	75	70.50	3.96					
6	300	310	305.00	45	52	48.50	3.98					
ogg1rad14												
1	460	400	430.00	41	46	43.50	2.53	2.17	1.36	**1.86**	**1.28**	**2.40**
2	520	550	535.00	35	38	36.50	1.71					
3	500	480	490.00	37	42	39.50	2.02					
4	520	400	460.00	20	22	21.00	1.14					
5	590	690	640.00	111	129	120.00	4.69					
6	560	580	570.00	21	23	22.00	0.96					

A III.a.3

Exp. 3.3

Frequencies of Spontaneous Can^S to Can^R mutation

Wild type

	YNBD plates (colonies $X10^{-5}$)/100μl			YNBD + CAN plates colonies/200μl			$can^R/10^6$	$can^+/10^6$				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEVE	Median	LIQV	UIQV
1	89	85	87.00	10	24	17.00	0.98	0.45	0.27	**0.36**	**0.31**	**0.42**
2	90	64	77.00	1	9	5.00	0.32					
3	97	70	83.50	6	2	4.00	0.24					
4	83	68	75.50	10	3	6.50	0.43					
5	103	86	94.50	9	6	7.50	0.40					
6	88	92	90.00	5	6	5.50	0.31					
ogg1												
1	89	106	97.50	38	29	33.50	1.72	3.04	0.98	**3.28**	**2.33**	**3.92**
2	83	65	74.00	46	51	48.50	3.28					
3	59	108	83.50	64	67	65.50	3.92					
4	75	60	67.50	23	40	31.50	2.33					
5	46	57	51.50	39	42	40.50	3.93					
rad14												
1	92	62	77.00	35	29	32.00	2.08	2.55	1.34	**2.04**	**1.95**	**2.31**
2	78	89	83.50	34	34	34.00	2.04					
3	80	111	95.50	32	43	37.50	1.96					
4	83	93	88.00	29	39	34.00	1.93					
5	138	105	121.50	135	134	134.50	5.53					
6	89	199	144.00	45	58	51.50	1.79					
7	99	94	96.50	47	51	49.00	2.54					
ogg1rad14												
1	108	113	110.50	50	48	49.00	2.22	1.99	0.56	**2.22**	**1.49**	**2.40**
2	93	124	108.50	63	35	49.00	2.26					
3	104	100	102.00	23	29	26.00	1.27					
4	106	106	106.00	54	54	54.00	2.55					
5	100	81	90.50	55	41	48.00	2.65					
6	79	89	84.00	30	24	27.00	1.61					

A III.a.4

Exp. 3.4

Frequencies of Spontaneous Can^S to Can^R mutation

	YNBD plates (colonies X 10^{-5})/100μl			YNBD+CAN plates colonies/100μl			$can^+/10^6$	$can^+/10^6$				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEV	Median	LIQV	UIQV
Wild type												
1	147	109	128.00	3	3	3.00	0.23	0.17	0.14	**0.17**	**0.07**	**0.23**
2	117	106	111.50	0	0	0.00	0.00					
3	120	131	125.50	1	1	1.00	0.08					
4	132	135	133.50	1	4	2.50	0.19					
5	93	125	109.00	2	3	2.50	0.23					
6	145	112	128.50	10	1	5.50	0.43					
7	95	93	94.00	0	3	1.50	0.16					
8	131	142	136.50	1	0	0.50	0.04					
ogg1												
1	73	68	70.50	16	18	17.00	2.41	3.50	1.47	**3.27**	**2.38**	**4.37**
2	69	76	72.50	24	36	30.00	4.14					
3	174	101	137.50	31	23	27.00	1.96					
4	194	135	164.50	85	102	93.50	5.68					
5	134	127	130.50	54	62	58.00	4.44					
6	87	94	90.50	15	28	21.50	2.38					
rad14												
1	111	72	91.50	13	30	21.50	2.35	2.92	1.77	**2.24**	**1.88**	**3.81**
2	141	169	155.00	20	21	20.50	1.32					
3	93	140	116.50	29	19	24.00	2.06					
4	122	122	122.00	80	73	76.50	6.27					
5	100	72	86.00	11	8	9.50	1.10					
6	86	86	86.00	37	24	30.50	3.55					
7	68	69	68.50	29	34	31.50	4.60					
8	130	96	113.00	28	20	24.00	2.12					
ogg1rad14												
1	131	109	120.00	73	52	62.50	5.21	2.56	2.18	**1.52**	**1.27**	**3.38**
2	148	109	128.50	23	16	19.50	1.52					
3	109	113	111.00	12	8	10.00	0.90					
4	118	154	136.00	80	89	84.50	6.21					
5	104	133	118.50	11	26	18.50	1.56					
6	103	145	124.00	18	11	14.50	1.17					
7	89	138	113.50	9	22	15.50	1.37					

A III.a.5

Frequencies of Spontaneous Can^S to Can ^{R} mutation

	YNBD plates colonies x10^{-4}/100µl			YNBD+CAN plates colonies/400µl			Can+/10^6	Can+/10^6				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEV	Median	LIQV	UIQV
Exp. 3.5												
Wild type												
1	420	511	465.50	2	4	3.00	0.16	**0.19**	**0.02**	0.19	0.18	0.21
2	423	390	406.50	3	4	3.50	0.22					
3	334	350	342.00	4	1	2.50	0.18					
4	420	403	411.50	3	4	3.50	0.21					
5	395	400	397.50	3	3	3.00	0.19					
Wild type + pYSB10												
1	299	303	301.00	1	2	1.50	0.12	**0.23**	**0.11**	0.24	0.12	0.27
2	320	285	302.50	0	3	1.50	0.12					
3	333	300	316.50	4	2	3.00	0.24					
4	256	290	273.00	5	1	3.00	0.27					
5	295	303	299.00	6	3	4.50	0.38					
Exp. 3.5												
ogg1												
1	803	635	719.00	24	21	22.50	0.78	**1.33**	**0.69**	1.17	0.78	1.47
2	723	710	716.50	26	18	22.00	0.77					
3	493	542	517.50	53	48	50.50	2.44					
4	646	674	660.00	29	33	31.00	1.17					
5	517	540	528.50	34	28	31.00	1.47					
***ogg1* + pYSB10**												
1	549	608	578.50	4	6	5.00	0.22	**0.10**	**0.07**	0.06	0.05	0.12
2	603	651	627.00	2	4	3.00	0.12					
3	681	781	731.00	2	1	1.50	0.05					
4	618	682	650.00	0	2	1.00	0.04					
5	648	582	615.00	0	3	1.50	0.06					
Exp. 3.5												
rad14												
1	498	567	532.50	53	45	49.00	2.30	**2.69**	**0.55**	2.48	2.41	2.76
2	556	598	577.00	92	70	81.00	3.51					
3	571	644	607.50	51	68	59.50	2.45					
4	546	479	512.50	53	50	51.50	2.51					
***rad14* + pYSB10**												
1	312	320	316.00	30	21	25.50	2.02	**2.19**	**0.33**	2.02	1.98	2.26
2	310	321	315.50	25	32	28.50	2.26					
3	342	331	336.50	41	33	37.00	2.75					
4	290	295	292.50	24	22	23.00	1.97					
5	331	350	340.50	33	21	27.00	1.98					

A III.a.5 continued

	YNBD plates colonies x10^{-4}/100μl			YNBD+CAN plates colonies/400μl			Can+/10^6	Can+/10^6 Mean	STDEV	Median	LIQV	UIQV
plate	1	2	(1+2)/2	1	2	(1+2)/2						
Exp. 3.5												
ogg1rad14												
1	500	473	486.50	58	57	57.50	2.95	**2.91**	**0.97**	2.61	2.23	3.28
2	472	443	457.50	61	95	78.00	4.26					
3	477	476	476.50	39	43	41.00	2.15					
4	404	492	448.00	32	49	40.50	2.26					
ogg1rad14 **+ pYSB10**												
1	297	335	316.00	12	15	13.50	1.07	**1.84**	**0.75**	1.80	1.31	2.00
2	342	345	343.50	21	15	18.00	1.31					
3	349	343	346.00	46	37	41.50	3.00					
4	386	378	382.00	22	33	27.50	1.80					
5	295	254	274.50	21	23	22.00	2.00					

A III.a.6

Frequencies of Spontaneous Can^S to Can ^{R} mutation

	YNBD plates colonies x10^{-4}/100μl			YNBD+CAN plates colonies/400μl			Can+/10^6	Can+/10^6				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEV	Median	LIQV	UIQV
Exp. 3.6												
Wild type												
1	500	450	475.00	4	2	3.00	0.16	**0.24**	**0.06**	0.24	0.23	0.26
2	394	402	398.00	7	3	5.00	0.31					
3	385	390	387.50	2	5	3.50	0.23					
4	411	356	383.50	6	2	4.00	0.26					
5	398	421	409.50	3	5	4.00	0.24					
Wild type + pYSB10												
1	295	302	298.50	4	4	4.00	0.34	**0.28**	**0.06**	0.28	0.24	0.34
2	250	280	265.00	3	2	2.50	0.24					
3	302	331	316.50	1	6	3.50	0.28					
4	298	283	290.50	5	0	2.50	0.22					
5	311	285	298.00	7	1	4.00	0.34					
Exp. 3.6												
ogg1												
1	519	401	460.00	60	45	52.50	2.85	**1.49**	**0.99**	1.07	0.71	2.20
2	803	788	795.50	44	24	34.00	1.07					
3	511	525	518.00	40	51	45.50	2.20					
4	1150	1150	1150.00	33	25	29.00	0.63					
5	876	784	830.00	20	27	23.50	0.71					
***ogg1* + pYSB10**												
1	620	618	619.00	3	8	5.50	0.22	**0.16**	**0.07**	0.18	0.14	0.21
2	642	705	673.50	1	2	1.50	0.06					
3	578	478	528.00	4	2	3.00	0.14					
4	536	522	529.00	4	5	4.50	0.21					
5	600	509	554.50	3	5	4.00	0.18					
Exp. 3.6												
rad14												
1	501	588	544.50	57	52	54.50	2.50	**2.33**	**0.29**	2.34	2.15	2.50
2	550	695	622.50	43	55	49.00	1.97					
3	589	589	589.00	63	64	63.50	2.70					
4	625	582	603.50	54	50	52.00	2.15					
5	613	596	604.50	55	58	56.50	2.34					
***rad14* + pYSB10**												
1	295	311	303.00	25	31	28.00	2.31	**2.40**	**0.45**	2.31	2.31	2.74
2	298	290	294.00	40	28	34.00	2.89					
3	314	320	317.00	21	23	22.00	1.74					
4	325	293	309.00	28	29	28.50	2.31					
5	285	300	292.50	35	29	32.00	2.74					

A III.a.6 continued

	YNBD plates colonies x10^{-4}/100μl			YNBD+CAN plates colonies/400μl			Can+/10^6	Can+/10^6 Mean	STDEV	Median	LIQV	UIQV
plate	1	2	(1+2)/2	1	2	(1+2)/2						
Exp. 3.6												
ogg1rad14												
1	394	410	402.00	31	32	31.50	1.96	**2.26**	**1.27**	1.96	1.79	2.13
2	450	583	516.50	100	82	91.00	4.40					
3	572	440	506.00	17	24	20.50	1.01					
4	436	456	446.00	27	37	32.00	1.79					
5	431	425	428.00	34	39	36.50	2.13					
ogg1rad14 **+ pYSB10**												
1	347	326	336.50	20	38	29.00	2.15	**1.41**	**0.42**	1.26	1.19	1.27
2	323	346	334.50	18	16	17.00	1.27					
3	381	314	347.50	16	19	17.50	1.26					
4	372	366	369.00	17	18	17.50	1.19					
5	330	295	312.50	15	14	14.50	1.16					

A III.a.7

Frequencies of Spontaneous Can^S to Can^R mutation

	YNBD plates colonies x10^{-4}/100µl			YNBD+CAN plates colonies/400µl			$can^+/10^6$	$can^+/10^6$				
plate	1	2	(1+2)/2	1	2	(1+2)/2		Mean	STDEV	Median	LIQV	UIQV
Exp. 3.7												
Wild type												
1	399	400	399.50	5	7	6.00	0.38	**0.28**	**0.10**	0.23	0.23	0.38
2	403	358	380.50	2	10	6.00	0.39					
3	368	402	385.00	4	3	3.50	0.23					
4	392	384	388.00	2	5	3.50	0.23					
5	410	401	405.50	4	1	2.50	0.15					
Wild type + pYSB10												
1	258	260	259.00	3	1	2.00	0.19	**0.23**	**0.03**	0.25	0.21	0.26
2	312	300	306.00	0	6	3.00	0.25					
3	295	293	294.00	4	2	3.00	0.26					
4	286	301	293.50	2	3	2.50	0.21					
5	295	292	293.50	5	1	3.00	0.26					
Exp. 3.7												
ogg1												
1	484	459	471.50	48	50	49.00	2.60	**2.26**	**0.76**	2.61	2.29	2.65
2	429	409	419.00	50	39	44.50	2.66					
3	454	459	456.50	44	36	40.00	2.19					
4	528	489	508.50	20	11	15.50	0.76					
5	490	469	479.50	45	59	52.00	2.71					
6	502	644	573.00	55	65	60.00	2.62					
***ogg1*+ pYSB10**												
1	506	418	462.00	3	3	3.00	0.16	**0.10**	**0.06**	0.09	0.08	0.14
2	330	311	320.50	2	0	1.00	0.08					
3	433	417	425.00	3	0	1.50	0.09					
4	285	308	296.50	1	1	1.00	0.08					
5	372	504	438.00	4	2	3.00	0.17					
6	416	524	470.00	0	1	0.50	0.03					
Exp. 3.7												
rad14												
1	600	569	584.50	54	49	51.50	2.20	**2.25**	**0.17**	2.24	2.20	2.35
2	559	600	579.50	54	55	54.50	2.35					
3	531	499	515.00	43	39	41.00	1.99					
4	564	530	547.00	56	51	53.50	2.45					
5	542	550	546.00	45	53	49.00	2.24					
***rad14* + pYSB10**												
1	331	350	340.50	34	25	29.50	2.17	**2.27**	**0.20**	2.28	2.17	2.43
2	285	301	293.00	27	30	28.50	2.43					
3	322	330	326.00	24	28	26.00	1.99					
4	281	295	288.00	25	32	28.50	2.47					
5	305	320	312.50	33	24	28.50	2.28					

A III.a.7 continued

	YNBD plates colonies $^{x}10^{-4}/100_{\mu}l$			YNBD+CAN plates colonies/$400_{\mu}l$			can+/10^6	Can+/10^6 Mean	STDEV	Median	LIQV	UIQV
plate	1	2	(1+2)/2	1	2	(1+2)/2						
Exp. 3.7												
ogg1rad14												
1	574	548	561.00	36	41	38.50	1.72	**1.66**	**0.82**	1.40	1.31	1.65
2	507	510	508.50	26	11	18.50	0.91					
3	467	459	463.00	56	64	60.00	3.24					
4	490	477	483.50	27	28	27.50	1.42					
5	503	441	472.00	26	26	26.00	1.38					
6	473	441	457.00	21	26	23.50	1.29					
***ogg1rad14*+ pYSB10**												
1	356	327	341.50	14	6	10.00	0.73	**1.12**	**1.02**	0.69	0.68	0.73
2	327	291	309.00	6	11	8.50	0.69					
3	308	315	311.50	7	10	8.50	0.68					
4	336	407	371.50	9	7	8.00	0.54					
5	350	271	310.50	31	42	36.50	2.94					

Appendix III.b

Statistical analysis of Chapter 3 data by Median test

A III.b.1

Exp. 3.1

	can$^+$/10^6			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.42	1.37	1.63	3.28
	0.15	3.07	3.34	1.02
	0.17	1.33	1.21	1.59
	0.21	1.36	2.24	0.88
	0.21	1.41	1.40	0.51
		1.41	0.90	2.45
Median	0.21	1.39	1.52	1.31
LIQV	0.17	1.36	1.26	0.92
UIQV	0.21	1.41	2.09	2.24

analysed with: Analyse-It + General v1.32

Test | **Median test**
Exp. 3.1
can$^+$/10^6: *ogg1, rad14, ogg1rad14*

n | 18

		Overall median = 1.405		
Exp. 3.1	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	6	3	3	1.390
rad14	6	3	3	1.515
ogg1rad14	6	3	3	1.305

p | 1.0000

A III.b.2

Exp. 3.2

	$can^+/10^6$			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.82	5.22	2.25	2.53
	0.67	4.42	2.87	1.71
	0.74	3.22	1.65	2.02
	0.50	3.28	2.59	1.14
	0.48	2.61	3.96	4.69
	0.13		3.98	0.96
Median	0.59	3.28	2.73	1.87
LIQV	0.49	3.22	2.34	1.28
UIQV	0.72	4.42	3.69	2.40

analysed with: Analyse-It + General v1.32

Test | **Median test**
Exp. 3.2
$Can^+/10^6$: *ogg1, rad14, ogg1rad14*

n | 17.00

		2.61		
Exp. 3.2	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	5.00	1.00	4.00	3.28
rad14	6.00	3.00	3.00	2.73
ogg1rad14	6.00	5.00	1.00	1.87

p | 0.11

A III.b.3

Exp. 3.3

	Can$^+$/10^6			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.98	1.72	2.08	2.22
	0.32	3.28	2.04	2.26
	0.24	3.92	1.96	1.27
	0.43	2.33	1.93	2.55
	0.4	3.93	5.53	2.65
	0.31		1.79	1.61
			2.54	1.38
Median	0.36	3.28	2.04	2.22
LIQV	0.31	2.33	1.95	1.50
UIQV	0.42	3.92	2.31	2.41

analysed with: Analyse-It + General v1.32

Test | **Median test**
Exp. 3.3
can+/10^6: *ogg1, rad14, ogg1rad14*

n | 19 (cases excluded: 3 due to missing values)

		Overall median = 2.22		
Exp. 3.3	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	5	1	4	3.28
rad14	7	5	2	2.04
ogg1rad14	7	4	3	2.22

p | 0.2034

Exp. 3.4

	$can^+/10^6$			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.23	2.41	2.35	5.21
	0	4.14	1.32	1.52
	0.08	1.96	2.06	0.9
	0.19	5.68	6.27	6.21
	0.23	4.44	1.1	1.56
	0.43	2.38	3.55	1.17
	0.16		4.6	1.37
	0.04		2.12	
Median	0.18	3.28	2.24	1.52
LIQV	0.07	2.39	1.88	1.27
UIQV	0.23	4.37	3.81	3.39

analysed with: Analyse-It + General v1.32

Test | **Median test**

Exp. 3.4

$Can^+/10^6$ *ogg1, rad14, ogg1rad14*

n | 21

		Overall median = 2.35		
Exp. 3.4	**n**	**≤** (Less or equal)	**>** (Greater)	**Median**
ogg1	6	1	5	3.28
rad14	8	5	3	2.24
ogg1rad14	7	5	2	1.52

p | 0.1100

A III.b.5

Exp. 3.5

Can$^+$/10^6

	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.16	0.78	2.3	2.95
	0.22	0.77	3.51	4.26
	0.18	2.44	2.45	2.15
	0.21	1.17	2.51	2.26
	0.19	1.47		
Median	0.19	1.17	2.48	2.61
LIQV	0.18	0.78	2.41	2.23
UIQV	0.21	1.47	2.76	3.28

analysed with: Analyse-It + General v1.32

Test | **Median test**
Exp. 3.5
Can$^+$/10^6: *ogg1, rad14, ogg1rad14*

n | 13

		Overall median = 2.3		
Exp..3.5	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	5	4	1	1.17
rad14	4	1	3	2.48
ogg1rad14	4	2	2	2.61

p | 0.2542

Exp. 3.6

	$Can^+/10^6$			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.16	2.85	2.5	1.96
	0.31	1.07	1.97	4.4
	0.23	2.2	2.7	1.01
	0.26	0.63	2.15	1.79
	0.24	0.71	2.34	2.13
Median	0.24	1.07	2.34	1.96
LIQV	0.23	0.71	2.15	1.79
UIQV	0.26	2.2	2.5	2.13

analysed with: Analyse-It + General v1.32

Test	**Median test**
	Exp. 3.6
	$Can^+/10^6$: *ogg1, rad14, ogg1rad14*

n	15

		Overall median = 2.13		
Exp..3.6	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	5	3	2	1.07
rad14	5	1	4	2.34
ogg1rad14	5	4	1	1.96

p	0.1534

A III.b.7

Exp. 3.7

	$Can^+/10^6$			
	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.38	2.6	2.2	1.72
	0.39	2.66	2.35	0.91
	0.23	2.19	1.99	3.24
	0.23	0.76	2.45	1.42
	0.15	2.71	2.24	1.38
		2.62		1.29
Median	0.23	2.61	2.24	1.40
LIQV	0.23	2.29	2.20	1.31
UIQV	0.38	2.65	2.35	1.65

analysed with: Analyse-It + General v1.32

Test | **Median test**
Exp. 3.7
$Can^+/10^6$: *ogg1, rad14, ogg1rad14*

n | 17

		Overall median = 2.2		
Exp..3.7	**n**	**≤** (Less or equal)	**>** (Greater)	**Median**
ogg1	6	2	4	2.61
rad14	5	2	3	2.24
ogg1rad14	6	5	1	1.40

p | 0.1749

A III.b.8

Ogg1

can$^+$/10^6

	Exp. 3.5		Exp. 3.6 Plasmid pYSB10		Exp. 3.7	
	-	+	-	+	-	+
	0.78	0.22	2.85	0.22	2.6	0.16
	0.77	0.12	1.07	0.06	2.66	0.08
	2.44	0.05	2.2	0.14	2.19	0.09
	1.17	0.04	0.63	0.21	0.76	0.08
	1.47	0.06	0.71	0.18	2.71	0.17
					2.62	0.03
Median	1.17	0.06	1.07	0.18	2.61	0.09
LIQV	0.78	0.05	0.71	0.14	2.29	0.08
UIQV	1.47	0.12	2.20	0.21	2.65	0.14

analysed with: Analyse-It + General v1.32

Test Median test

Can$^+$/10^6

overexpression of *OGG1* in the *ogg1* mutant

n	10

Overall median = 0.495

Exp. 3.5	n	≤ (Less or equal)	> (Greater)	Median
ogg1	5	0	5	1.17
***ogg1*+ pYSB10**	5	5	0	0.06

p	0.0079 (exact, double 1-tailed p)

n	10

Overall median = 0.425

Exp. 3.6	n	≤ (Less or equal)	> (Greater)	Median
ogg1	5	0	5	1.07
***ogg1*+ pYSB10**	5	5	0	0.18

p	0.0079 (exact, double 1-tailed p)

n	12

Overall median = 0.465

Exp. 3.7	n	≤ (Less or equal)	> (Greater)	Median
ogg1	6	0	6	2.61
***ogg1*+ pYSB10**	6	6	0	0.09

p	0.0022 (exact, double 1-tailed p)

rad14

Can$^+$/10^6

Exp. 3.5		Exp. 3.6		Exp. 3.7	
Plasmid pYSB10					
-	+	-	+	-	+
2.3	2.02	2.5	2.31	2.2	2.17
3.51	2.26	1.97	2.89	2.35	2.43
2.45	2.75	2.7	1.74	1.99	1.99
2.51	1.97	2.15	2.31	2.45	2.47
	1.98	2.34	2.74	2.24	2.28
Median	2.48	2.02	2.34	2.31	2.24	2.28
LIQV	2.4125	1.98	2.15	2.31	2.2	2.17
UIQV	2.76	2.26	2.5	2.74	2.35	2.43

analysed with: Analyse-It + General v1.32

Test	**Median test**
	can$^+$/10^6
	Overexpression of *OGG1* in the *rad14* mutant

n	9

		Overall median = 2.3		
Exp. 3.5	**n**	**≤ (Less or equal)**	**> (Greater)**	**Median**
rad14	4	1	3	2.48
***rad14* + pYSB10**	5	4	1	2.02

p	0.3333 (exact, double 1-tailed p)

n	10

		Overall median = 2.325		
Exp.3.6	**n**	**≤ (Less or equal)**	**> (Greater)**	**Median**
rad14	5	2	3	2.34
***rad14* + pYSB10**	5	3	2	2.31

p	1.0000 (exact, double 1-tailed p)

n	10

		Overall median = 2.26		
Exp.3.7	**n**	**≤ (Less or equal)**	**> (Greater)**	**Median**
rad14	5	3	2	2.24
***rad14*+ pYSB10**	5	2	3	2.28

p	1.0000 (exact, double 1-tailed p)

A III.b.10

ogg1rad14

$Can^+/10^6$

	Exp. 3.5		Exp. 3.6		Exp. 3.7	
	Plasmid pYSB10					
	-	+	-	+	-	+
	2.95	1.07	1.96	2.15	1.72	0.73
	4.26	1.31	4.4	1.27	0.91	0.69
	2.15	3	1.01	1.26	3.24	0.68
	2.26	1.8	1.79	1.19	1.42	0.54
		2	2.13	1.16	1.38	2.94
					1.29	
Median	2.605	1.80	1.96	1.26	1.40	0.69
LIQV	2.2325	1.31	1.79	1.19	1.31	0.68
UIQV	3.2775	2.00	2.13	1.27	1.65	0.73

analysed with: Analyse-It + General v1.40

Test | **Median test**
$Can^+/10^6$
Overexpression of *OGG1* in the *ogg1rad14* mutant

n | 9

Overall median = 2.15

Exp.3.5	n	≤ (Less or equal)	> (Greater)	Median
ogg1rad14	4	1	3	2.61
***ogg1rad14*+pYSB10**	5	4	1	1.80

p | 0.3333 (exact, double 1-tailed p)

n | 10

Overall median = 1.53

Exp.3.6	n	≤ (Less or equal)	> (Greater)	Median
ogg1rad14	5	1	4	1.96
***ogg1rad14*+pYSB10**	5	4	1	1.26

p | 0.2063 (exact, double 1-tailed p)

n | 11

Overall median = 1.29

Exp.3.7	n	≤ (Less or equal)	> (Greater)	Median
ogg1rad14	6	2	4	1.40
***ogg1rad14*+pYSB10**	5	4	1	0.69

p | 0.3506 (exact, double 1-tailed p)

A III.b.11

Summary of of the data for experiments 3.1-3.7 for each strain

	Can$^+$/10^6			
	wild type	*ogg1*	*rad14*	*gg1rad14*
	0.42	1.37	1.63	3.28
	0.15	3.07	3.34	1.02
	0.17	1.33	1.21	1.59
	0.21	1.36	2.24	0.88
	0.21	1.41	1.4	0.51
	0.82	1.41	0.9	2.45
	0.67	5.22	2.25	2.53
	0.74	4.42	2.87	1.71
	0.5	3.22	1.65	2.02
	0.48	3.28	2.59	1.14
	0.13	2.61	3.96	4.69
	0.98	1.72	3.98	0.96
	0.32	3.28	2.08	2.22
	0.24	3.92	2.04	2.26
	0.43	2.33	1.96	1.27
	0.4	3.93	1.93	2.55
	0.31	2.41	5.53	2.65
	0.23	4.14	1.79	1.61
	0	1.96	2.54	1.38
	0.08	5.68	2.35	5.21
	0.19	4.44	1.32	1.52
	0.23	2.38	2.06	0.9
	0.43	0.78	6.27	6.21
	0.16	0.77	1.1	1.56
	0.04	2.44	3.55	1.17
	0.16	1.17	4.6	1.37
	0.22	1.47	2.12	2.95
	0.18	2.85	2.3	4.26
	0.21	1.07	3.51	2.15
	0.19	2.2	2.45	2.26
	0.16	0.63	2.51	1.96
	0.31	0.71	2.5	4.4
	0.23	2.6	1.97	1.01
	0.26	2.66	2.7	1.79
	0.24	2.19	2.15	2.13
	0.38	0.76	2.34	1.72
	0.39	2.71	2.2	0.91
	0.23	2.62	2.35	3.24
	0.23		1.99	1.42
	0.15		2.45	1.38
			2.24	1.29
Median	0.23	2.40	2.25	1.72
LIQV	0.18	1.38	1.97	1.29
UIQV	0.39	3.18	2.59	2.53

A III.b.11 continued

analysed with: Analyse-It + General v1.40

Test | **Median test**
Can^{+}/10^{6}
Exp. 3.1-3.7

Exp. 3.1-3.7 n | 78

Overall median = 0.725

	n	≤ (Less or equal)	> (Greater)	Median
wild type	40	37	3	0.23
ogg1	38	2	36	2.40

p | <0.0001 (exact, double 1-tailed p)

Exp. 3.1-3.7 n | 81

Overall median = 0.98

	n	≤ (Less or equal)	> (Greater)	Median
wild type	40	40	0	0.23
rad14	41	1	40	2.25

p | <0.0001 (exact, double 1-tailed p)

Exp. 3.1-3.7 n | 81

Overall median = 0.88

	n	≤ (Less or equal)	> (Greater)	Median
wild type	40	39	1	0.23
ogg1rad14	41	2	39	1.72

p | <0.0001 (exact, double 1-tailed p)

Exp. 3.1-3.7 n | 120

Overall median = 2.2

	n	≤ (Less or equal)	> (Greater)	Median
ogg1	38	17	21	2.40
rad14	41	18	23	2.25
ogg1rad14	41	26	15	1.72

p | 0.1388

A III.b.12

Summary of the data for experiments 3.5-3.7 for each strain

	wild type		*ogg1*		*rad14*		*ogg1rad14*	
	plasmid-	plasmid+	plasmid-	plasmid+	plasmid-	plasmid+	plasmid-	plasmid+
	0.16	0.12	0.78	0.22	2.3	2.02	2.95	1.07
	0.22	0.12	0.77	0.12	3.51	2.26	4.26	1.31
	0.18	0.24	2.44	0.05	2.45	2.75	2.15	3
	0.21	0.27	1.17	0.04	2.51	1.97	2.26	1.8
	0.19	0.38	1.47	0.06	2.5	1.98	1.96	2
	0.16	0.34	2.85	0.22	1.97	2.31	4.4	2.15
	0.31	0.24	1.07	0.06	2.7	2.89	1.01	1.27
	0.23	0.28	2.2	0.14	2.15	1.74	1.79	1.26
	0.26	0.22	0.63	0.21	2.34	2.31	2.13	1.19
	0.24	0.34	0.71	0.18	2.2	2.74	1.72	1.16
	0.38	0.19	2.6	0.16	2.35	2.17	0.91	0.73
	0.39	0.25	2.66	0.08	1.99	2.43	3.24	0.69
	0.23	0.26	2.19	0.09	2.45	1.99	1.42	0.68
	0.23	0.21	0.76	0.08	2.24	2.47	1.38	0.54
	0.15	0.26	2.71	0.17		2.28	1.29	2.94
			2.62	0.03				
MEDIAN	0.230	0.250	1.830	0.105	2.345	2.280	1.960	1.260
LIQV	0.185	0.215	0.778	0.060	2.210	2.005	1.400	0.900
UIQV	0.250	0.275	2.605	0.175	2.488	2.450	2.605	1.900

A III.b.12 continued

analysed with: Analyse-It + General v1.40

Test | **Median test**
$Can^+/10^6$

Exp. 3.5-3.7 n | 30

Overall median = 0.235

wild type	n	≤ (Less or equal)	> (Greater)	Median
plasmid-	15	10	5	0.23
plasmid+	15	5	10	0.25

p | 0.1431 (exact, double 1-tailed p)

Exp. 3.5-3.7 n | 32

Overall median = 0.425

ogg1	n	≤ (Less or equal)	> (Greater)	Median
plasmid-	16	0	16	1.83
plasmid+	16	16	0	0.11

p | <0.0001 (exact, double 1-tailed p)

Exp. 3.5-3.7 n | 29

Overall median = 2.31

rad14	n	≤ (Less or equal)	> (Greater)	Median
plasmid-	14	6	8	2.35
plasmid+	15	10	5	2.28

p | 0.3609 (exact, double 1-tailed p)

Exp. 3.5-3.7 n | 30

Overall median = 1.57

ogg1rad14	n	≤ (Less or equal)	> (Greater)	Median
plasmid-	15	5	10	1.96
plasmid+	15	10	5	1.26

p | 0.1431 (exact, double 1-tailed p)

Appendix IV (Data for Chapter 4)

Appendix IV.a (Data and basic statistics)

A IV.a.1

wild type-Expt. 4.1

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	79	75	77	1	2	1.5	0.02
2	54	72	63	2	1	1.5	0.02
3	80	81	80.5	3	2	2.5	0.03
4	90	85	87.5	2	2	2	0.02
5	86	75	80.5	1	3	2	0.02
						Median	0.02
						LIQV	0.02
						UIQV	0.02

rad14*-Expt. *4.1

	glucose$^+$plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	71	61	66	3	0	1.5	0.02
2	68	69	68.5	0	3	1.5	0.02
3	90	86	88	4	1	2.5	0.03
4	75	80	77.5	2	1	1.5	0.02
5	92	88	90	2	2	2	0.02
						Median	0.02
						LIQV	0.02
						UIQV	0.02

A IV.a.1 (continued)

ogg1-Expt. 4.1

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	coloniesx10				mean	$YC^+/10^7$
1	72	86	79	2	2	0	0	1.00	0.13
2	104	104	104	3	2	0	0	1.25	0.12
3	47	31	39	0	1	0	0	0.25	0.06
4	118	111	114.5	1	2	0	2	1.25	0.11
5	57	65	61	1	2	1	0	1.00	0.16
6	133	89	111	2	1	1	0	1.00	0.09
7	120	90	105	0	0	2	0	0.50	0.05
8	97	126	111.5	1	1	2	0	1.00	0.09
								Median	**0.10**
								LIQV	**0.08**
								UIQV	**0.12**

ogg1rad14-Expt. 4.1

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	coloniesx10				mean	$YC^+/10^7$
1	83	77	80	25	23	28	18	23.50	2.94
2	85	85	85	4	0	0	3	1.75	0.21
4	112	98	105	59	28	36	65	47.00	4.48
5	48	35	41.5	3	4	3	1	2.75	0.66
6	105	109	107	15	37	50		34.00	3.18
8	125	116	120.5	1	1	1	2	1.25	0.10
9	97	87	92	45	25	41	51	40.50	4.40
								Median	**2.94**
								LIQV	**0.43**
								UIQV	**3.79**

A IV.a.2

wild type-Expt.4.2

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	87	64	75.5	2	2	2	0.03
2	83	87	85	4	2	3	0.04
3	84	56	70	3	0	1.5	0.02
4	95	86	90.5	3	4	3.5	0.04
5	79	80	79.5	2	4	3	0.04
						Median	0.04
						LIQV	0.03
						UIQV	0.04

rad14*- Expt. *4.2

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	82	81	81.5	4	4	4	0.05
2	74	35	54.5	1	1	1	0.02
3	45	68	56.5	4	2	3	0.05
4	54	46	50	2	3	2.5	0.05
5	87	65	76	0	0	0	0.00
						Median	0.05
						LIQV	0.02
						UIQV	0.05

A IV.a.2 (continued)

ogg1- Expt. 4.2

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	coloniesx10				mean	YC$^+$/10^7
1	121	122	121.5	10	17	6	2	8.75	0.72
2	98	119	108.5	3	3	1	2	2.25	0.21
3	76	116	96	12	9	4	2	6.75	0.70
4	107	104	105.5	0	0	1	0	0.25	0.02
5	131	97	114	3	7	6	6	5.50	0.48
6	112	86	99	6	4	4		4.67	0.47
7	111	132	121.5	1	3	6		3.33	0.27
8	92	94	93	0	1	0	2	0.75	0.08
9	109	111	110	226	260	116		200.67	18.24
10	111	120	115.5	1	0	2		1.00	0.09
								Median	0.37
								LIQV	0.12
								UIQV	0.65

ogg1rad14- Expt. 4.2

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	coloniesx10				mean	YC$^+$/10^7
1	120	91	105.5	2172	1288			1730.00	163.98
2	70	55	62.5	1212	1128			1170.00	187.20
3	67	65	66	146	59	66	77	87.00	13.18
4	81	70	75.5	43	32	26	23	31.00	4.11
5	125	134	129.5	968				968.00	74.75
6	78	85	81.5	2232	3432			2832.00	347.48
7	85	76	80.5	141	137	69	56	100.75	12.52
8	68	61	64.5	16	18	13	16	15.75	2.44
9	120	140	130	145	117	171		144.33	11.10
10	122	127	124.5	11	10	10	9	10.00	0.80
								Median	**12.85**
								LIQV	**5.86**
								UIQV	**141.67**

A IV.a.3

wild type-Expt. 4.3

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	71	72	71.5	5	2	3.5	0.05
2	65	55	60	2	2	2	0.03
3	52	60	56	3	3	3	0.05
4	60	57	58.5	3	3	3	0.05
5	82	75	78.5	3	5	4	0.05
						Median	0.05
						LIQV	0.05
						UIQV	0.05

***rad14*- Expt. 4.3**

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	59	41	50	4	2	3	0.06
2	31	42	36.5	2	1	1.5	0.04
3	61	52	56.5	2	4	3	0.05
4	72	76	74	4	2	3	0.04
5	68	71	69.5	5	2	3.5	0.05
						Median	0.05
						LIQV	0.04
						UIQV	0.05

A IV.a.3 (continued)

ogg1- Expt. 4.3

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	colonies x10				mean	YC$^+$/10^7
1	138	141	139.5	7	2	9		6.00	0.43
2	160	177	168.5	9	12	10	4	8.75	0.52
3	150	123	136.5	5	0	0	0	1.25	0.09
4	150	155	152.5	5	3	3	18	7.25	0.48
5	172	170	171	4	14	20	8	11.50	0.67
6	173	147	160	575	628			601.50	37.59
7	146	182	164	18	11	12	6	11.75	0.72
8	135	136	135.5	13	7	8	17	11.25	0.83
9	143	140	141.5	3	1	3	0	1.75	0.12
10	118	152	135	5	10	2	0	4.25	0.31
								Median	**0.50**
								LIQV	**0.34**
								UIQV	**0.71**

ogg1rad14- Expt. 4.3

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	colonies x10				mean	YC$^+$/10^7
1	59	43	51	115	106	107	123	112.75	22.11
2	80	66	73	23	12	2	2	9.75	1.34
3	86	83	84.5	5	3	4	4	4.00	0.47
4	86	103	94.5	253	279	270	258	265.00	28.04
5	35	38	36.5	21	21	23	31	24.00	6.58
6	53	38	45.5	384	307	291		327.33	71.94
7	62	95	78.5	0	0	0	0	0.00	0.00
8	78	57	67.5	14	12			13.00	1.93
9	73	77	75	483	325	457	491	439.00	58.53
								Median	**6.58**
								LIQV	**1.34**
								UIQV	**28.04**

A IV.a.4

wild type-Expt. 4.4

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
Expt..4							
1	94	78	86	2	1	1.5	0.02
2	62	89	75.5	1	5	3	0.04
3	74	75	74.5	0	3	1.5	0.02
4	81	76	78.5	1	2	1.5	0.02
5	68	75	71.5	2	1	1.5	0.02
						Median	0.02
						LIQV	0.02
						UIQV	0.02

***rad14*-Expt. 4.4**

	glucose$^+$ plates			glucose$^-$ plates			
	colonies/10^5		mean	coloniesx100		mean	CYC$^+$/10^7
1	69	80	74.5	3	0	1.5	0.02
2	72	63	67.5	1	2	1.5	0.02
3	67	70	68.5	2	5	3.5	0.05
4	75	62	68.5	2	1	1.5	0.02
5	81	75	78	3	0	1.5	0.02
						Median	0.02
						LIQV	0.02
						UIQV	0.02

A IV.a.4 (continued)

ogg1- Expt. 4.4

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	colonies x10				mean	YC$^+$/10^7
1	116	100	108	1	0	1	0	0.50	0.05
2	117	134	125.5	0	0	0	0	0.00	0.00
3	110	113	111.5	0	0	3	0	0.75	0.07
4	142	112	127	3	0	4	5	3.00	0.24
5	137	148	142.5	1	0	1	2	1.00	0.07
6	126	142	134	142	113	88	70	103.25	7.71
7	140	110	125	1	1	5	2	2.25	0.18
8	159	111	135	1	5	1	1	2.00	0.15
9	140	153	146.5	1	1	1	4	1.75	0.12
10	143	126	134.5	1	0	1	0	0.50	0.04
								Median	**0.09**
								LIQV	**0.05**
								UIQV	**0.17**

ogg1rad14- Expt. 4.4

	glucose$^+$ plates			glucose$^-$ plates					
	colonies/10^5		mean	colonies x10				mean	YC$^+$/10^7
1	129	98	113.50	672				672.00	59.21
2	106	96	101.00	88	22	45		51.67	5.12
3	105	116	110.50	1356				1356.00	122.71
4	82	102	92.00	6	1	2	1	2.50	0.27
5	144	97	120.50	12	8	2	6	7.00	0.58
6	82	100	91.00	24	15	30	31	25.00	2.75
7	156	181	168.50	3	1	0	0	1.00	0.06
8	178	155	166.50	0	1	2	0	0.75	0.05
9	166	171	168.50	6	6	1	0	3.25	0.19
								Median	**0.58**
								LIQV	**0.19**
								UIQV	**5.12**

Appendix IV.b

Statistical analysis of Chapter 4 data by Median test

A IV b.1

Expt. 4.1

$CYC^+/10^7$

	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.02	0.13	0.02	2.94
	0.02	0.12	0.02	0.21
	0.03	0.06	0.03	4.48
	0.02	0.11	0.02	0.66
	0.02	0.16	0.02	3.18
		0.09		0.10
		0.05		4.40
		0.09		
Median	0.02	0.10	0.02	2.94
LIQV	0.02	0.08	0.02	0.44
UIQV	0.02	0.12	0.02	3.79

analysed with: Analyse-It + General v1.32

Test | **Median test**
Expt.4.1
$CYC^+/10^7$: *ogg1, ogg1rad14*

		Overall median = 0.13		
CYC+/107	**n**	**≤** (Less or equal)	**>** (Greater)	**Median**
ogg1	8	7	1	0.10
ogg1rad14	7	1	6	2.94

p | 0.0177 (exact, double 1-tailed p)

Expt. 4.2

$CYC^+/10^7$

	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.03	0.72	0.05	163.98
	0.04	0.21	0.02	187.20
	0.02	0.70	0.05	13.18
	0.04	0.02	0.05	4.11
	0.04	0.48	0.00	74.75
		0.47		347.48
		0.27		12.52
		0.08		2.44
		18.24		11.10
		0.09		0.80
Median	0.04	0.37	0.05	12.85
LIQV	0.03	0.12	0.02	5.86
UIQV	0.04	0.65	0.05	141.67

analysed with: Analyse-It + General v1.32

Test | **Median test**
Expt.4.2
$CYC^+/10^7$: *ogg1, ogg1rad14*

n | 20

		Overall median = 1.62		
CYC+/107	**n**	**≤ (Less or equal)**	**> (Greater)**	**Median**
ogg1	10	9	1	0.37
ogg1rad14	10	1	9	12.85

p | 0.0011 (exact, double 1-tailed p)

Expt. 4.3

$CYC^+/10^7$

	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.05	0.43	0.06	22.11
	0.03	0.52	0.04	1.34
	0.05	0.09	0.05	0.47
	0.05	0.48	0.04	28.04
	0.05	0.67	0.05	6.58
		37.59		71.94
		0.72		0.00
		0.83		1.93
		0.12		58.53
		0.31		
Median	0.05	0.50	0.05	6.58
LIQV	0.05	0.34	0.04	1.34
UIQV	0.05	0.71	0.05	28.04

analysed with: Analyse-It + General v1.32

Test | **Median test**
Expt.4.3
$CYC^+/10^7$: *ogg1, ogg1rad14*

n | 19

		Overall median = 0.72		
CYC+/107	**n**	**≤** (Less or equal)	**>** (Greater)	**Median**
ogg1	10	8	2	0.50
ogg1rad14	9	2	7	6.58

p | 0.0370 exact double one tailed p

Expt. 4.4

$CYC^+/10^7$

	wild type	*ogg1*	*rad14*	*ogg1rad14*
	0.02	0.05	0.02	59.21
	0.04	0.00	0.02	5.12
	0.02	0.07	0.05	122.71
	0.02	0.24	0.02	0.27
	0.02	0.07	0.02	0.58
		7.71		2.75
		0.18		0.06
		0.15		0.05
		0.12		0.19
		0.04		
Median	0.02	0.10	0.02	0.58
LIQV	0.02	0.06	0.02	0.19
UIQV	0.02	0.17	0.02	5.12

analysed with: Analyse-It + General v1.32

Test | **Median test**
Expt.4.4
$CYC^+/10^7$: *ogg1,ogg1rad14*

n | 19

		Overall median = 0.18		
CYC+/107	**n**	≤ (Less or equal)	> (Greater)	**Median**
ogg1	10	8	2	0.095
ogg1rad14	9	2	7	0.580

p | 0.0370 (exact, double one tailed p)

Appendix IV.b.5

Summary of the data for experiments 4.1-4.4

CYC$^+$/10^7			
wild type	*ogg1*	*rad14*	*ogg1rad14*
0.02	0.13	0.02	2.94
0.02	0.12	0.02	0.21
0.03	0.06	0.03	4.48
0.02	0.11	0.02	0.66
0.02	0.16	0.02	3.18
0.03	0.09	0.05	0.1
0.04	0.05	0.02	4.4
0.02	0.09	0.05	163.98
0.04	0.72	0.05	187.2
0.04	0.21	0	13.18
0.05	0.7	0.06	4.11
0.03	0.02	0.04	74.75
0.05	0.48	0.05	347.48
0.05	0.47	0.04	12.52
0.05	0.27	0.05	2.44
0.02	0.08	0.02	11.1
0.04	18.24	0.02	0.8
0.02	0.09	0.05	22.11
0.02	0.43	0.02	1.34
0.02	0.52	0.02	0.47
	0.09		28.04
	0.48		6.58
	0.67		71.94
	37.59		0
	0.72		1.93
	0.83		58.53
	0.12		59.21
	0.31		5.12
	0.05		122.71
	0		0.27
	0.07		0.58
	0.24		2.75
	0.07		0.06
	7.71		0.05
	0.18		0.19
	0.15		
	0.12		
	0.04		
Median 0.03	0.155	0.025	4.11
LIQV 0.02	0.09	0.02	0.62
UIQV 0.04	0.48	0.05	25.075

Appendix IV.b.5 continued

analysed with: Analyse-It + General v1.32

Test | **Median test**
Sum of experiments 4.1-4.4
$CYC^+/10^7$: wild type, *rad14*

n | 40

		Overall median = 0.03		
$CYC+/10^7$	n	≤ (Less or equal)	> (Greater)	Median
wild type	20	12	8	0.030
rad14	20	11	9	0.025

p | 1.0000 (exact, double 1-tailed p)

analysed with: Analyse-It + General v1.32

Test | **Median test**
Sum of experiments 4.1-4.4
$CYC^+/10^7$: *ogg1*, *ogg1rad14*

n | 73

		Overall median = 0.48		
$CYC+/10^7$	n	≤ (Less or equal)	> (Greater)	Median
ogg1	38	29	9	0.155
ogg1rad14	35	8	27	4.110

p | <0.0001 (exact, double 1-tailed p)

Appendix V

Description of the Statistical Analysis terms

Mean: The average of a set of numbers.

Standard deviation (STDEV): A Measure of how widely values are dispersed from the average or mean value.

Median: The number in the middle of a set of numbers; that is, the half of the numbers have values that are greater than the median, and half have values that are less. If there is an even number of numbers in a set, the median calculates the average of the two numbers in the middle.

Upper and lower inter-quartile values (UIQV, LIQV): The 75^{th} and 25^{th} percentile values in a population divided into 4 sections between the Minimum value , LIQV (25^{th} percentile value), Median (50^{th} percentile value), UIQV (75^{th} percentile value), and the Maximum value.

Median Test

A median test determines whether the null hypothesis, which states there is no significant difference between the medians, is true for two or more data sets under question. In this test a predetermined probability value (P) (taken here as 0.05) determines the outcome of the test. If $P>0.05$ the null hypothesis is accepted; i.e. there is no significant difference between the medians. However, If $P\leq0.05$, the null hypothesis is rejected; i.e. there is a significant difference between the medians. The smaller the P value, the more untenable the null hypothesis. The median test is a suitable test for statistical analysis of the data obtained from spontaneous mutation analysis because the samples obtained form such experiments do not have a similar shape distribution and they represent a very high standard deviation. Furthermore, unlike the mean tests, the extreme values (very low or very high) in the population, do not dramatically effect the outcome of a median test.

Calculation tool for statistical analysis of the data

Microsoft Excel (version 97) and Analyse-it for Microsoft Excel, from Analyse-it software LTD, downloaded from *web: http//www.analyse-it .com*, was used for the statistical analysis of the data in chapter 3 and 4 of this thesis.

References

Aboussekhra, A., Biggerstaff, M., Shivji, M. K. K., Vilpo, J. A., Moncollin, V., Podust, V. N., Protic, M., Hubscher, U., Egly, J. M., and Wood, R. D. (1995). Mammalian DNA Nucleotide Excision Repair Reconstituted with Purified Protein Components. Cell 80, 859-868.

Ames, B. N. (1989). Mutagenesis and carcinogenesis: Endogenous and exogenous factors. Environmental and Molecular Mutagenesis 14, 66-77.

Ames, B. N., and Whitfield, H. J. (1966). Frameshift mutagenesis in Salmonella. Cold Spring Harbour Symposium on Quantitative Biology 31, 221-225.

Ames, B. N., Shigenaga, M. K., and Gold, L. S. (1993a). DNA lesions, inducible DNA repair, and cell division: Three key factors in mutagenesis and carcinogenesis. Environmental Health Perspectives 101, 35-44.

Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993b). Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA 90, 7915-7922.

Araújo, S.J. and Wood, R. (1999). Protein complexes in nucleotide excision repair. Mutation research. 435, 23-33.

Aruoma, O. I., Halliwell, B., and Dizdaroglu, M. (1989). Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. J. Biol. Chem. 264, 13024-13028.

Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., and Cunningham, R. P. (1989). Purification and characterisation of *E. coli* endonuclease III from the cloned *NTH* gene. Biochemistry 28, 4444-4449.

Asahina, H., Kuraoka, I., Shirakawa, M., Morita, E. H., Miura, N., Miyamoto, I., Ohtsuka, E., Okada, Y., and Tanaka, K. (1994). The XPA protein is a zinc metalloprotein with an ability to recognize various kinds of DNA damage. Mutation Research - DNA Repair 315, 229-237.

Augeri, L., Lee, Y. M., Barton, A. B., and Doetsch, P. W. (1997). Purification, characterization, gene cloning, and expression of *Saccharomyces cerevisiae* redoxyendonuclease, a homolog of *Escherichia coli* endonuclease III. Biochemistry 36, 721-729.

Badawi, A. F. (1996). Molecular and genetic events in schistosomiasis-associated human bladder-cancer - role of oncogenes and tumor-suppressor genes. Cancer Letters 105, 123-138.

Bailly, V. (1987). *Escherichia-coli* endonuclease-III and the repair of oxidized thymines and AP sites in DNA. Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales 181, 52-54.

Bankmann, M., Prakash, L., and Prakash, S. (1992). Yeast *RAD14* and human xeroderma pigmentosum group A DNA-repair genes encode homologous proteins. Nature 355, 555-558.

Bardwell, A. J., Bardwell, L., Iyer, N., Svejstrup, J. Q., Feaver, W. J., Kornberg, R. D., and Friedberg, E. C. (1994a). Yeast nucleotide excision-repair proteins Rad2 and Rad4 interact with RNA-polymerase-II basal transcription factor-b (TFIIH). Mol. Cell. Biol. 14, 3569-3576.

Bardwell, A. J., Bardwell, L., Wang, Z., Siede, W., Reagan, M. S., Tomkinson, A. E., Friedberg, A. S., Pittenger, C., Feaver, W. J., Svejstrup, J., Kornberg, R. D., and Friedberg, E. C. (1994b). Recent insights on DNA repair. The mechanism of damaged nucleotide excision in eukaryotes and its relationship to other cellular processes. Annals of the New York Academy of Sciences 726, 281-291.

Basu, A. K., Loechler, E. L., Leadon, S. A., and Essigmann, J. M. (1989). Genetic-effects of thymine glycol - site-specific mutagenesis and molecular modeling studies - (ionizing-radiation oxidative damage hydroxyl radicals). Proc. Natl. Acad. Sci. USA 86, 7677-7681.

Beckman, K. B., and Ames, B. N. (1997). Oxidative decay of DNA. J. Biol. Chem. 272, 19633-19636.

Bertrand, P., Tishkoff, D. X., Filosi, N., Dasgupta, R., and Kolodner, R. D. (1998). Physical interaction between components of DNA mismatch repair and nucleotide excision repair. Proc. Natl. Acad. Sci. USA 95, 14278-14283.

Bessho, T. (1999). Nucleotide excision repair 3' endonuclease XPG stimulates the activity of base excision repair enzyme thymine glycol DNA glycosylase. Nucleic Acids Res. 27, 979-983.

Bessho, T., Tano, K., Kasai, H., and Nishimura, S. (1992). Deficiency of 8-hydroxyguanine DNA endonuclease activity and accumulation of the 8-hydroxyguanine in mutator mutant (*mutM*) of *Escherichia coli*. Biochemical and Biophysical Research Communications 188, 372-378.

Bessho, T., Roy, R., Yamamoto, K., Kasai, H., Nishimura, S., Tano, K., and Mitra, S. (1993). Repair of 8-hydroxyguanine in DNA by mammalian N-methylpurine-DNA glycosylase. Proc. Natl. Acad. Sci. USA 90, 8901-8904.

Bhatia, P. K., Verhage, R. A., Brouwer, J., and Friedberg, E. C. (1996a). Molecular cloning and characterisation of *Saccharomyces cerevisiae RAD28*, the yeast homolog of the human Cockayne Syndrome A (CSA) gene. Journal of Bacteriology 178, 5977-5988.

Bhatia, P. K., Wang, Z. G., and Friedberg, E. C. (1996b). DNA-repair and transcription. Curr. Opin. Genet. Dev. 6, 146-150.

Bohr, V. A., and Hanawalt, P. C. (1987). Enhanced repair of pyrimidine dimers in coding and non-coding genomic sequences in CHO cells expressing a prokaryotic DNA-repair gene. Carcinogenesis 8, 1333-1336.

Bohr, V. A., Smith, C. A., Okumoto, D. S., and Hanawalt, P. C. (1985). DNA repair in an active gene: Removal of pyrimidine dimers from the *DHFR* gene of CHO cells is much more efficient than in the genome overall. Cell 40, 359-369.

Bohr, V. A., Phillips, D. H., and Hanawalt, P. C. (1987). Heterogeneous DNA damage and repair in the mammalian genome. Cancer Research 47, 6426-6436.

Bohr, V. A., Evans, M. K., and Fornace, A. J., Jr. (1989). DNA repair and its pathogenetic implications. Laboratory Investigation 61, 143-161.

Bohr, V., Anson, R. M., Mazur, S., and Dianov, G. (1998). Oxidative DNA damage processing and changes with aging. Toxicology Letters 103, 47-52.

Bohr, V. and Dianov, G., (1999) Oxidative DNA damage processing in nuclear and mitochondrial DNA. Biochimie 81, 155-160.

Boiteux, S. (1993). Properties and biological functions of the Nth and Fpg proteins of *Escherichia coli*: two DNA glycosylases that repair oxidative damage in DNA. Journal of Photochemistry and Photobiology B: Biology 19, 87-96.

Boiteux, S., and Laval, J. (1983). Imidazole open ring 7-methylguanine - an inhibitor of DNA-synthesis. Biochemical and Biophysical Research Communications 110, 552-558.

Boiteux, S., and Radicella, J. P. (1999). Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress. Biochimie 81, 59-67.

Boiteux, S., O'Connor, T. S., and Laval, J. (1987). Formamidopyrimidine-DNA glycosylase of *Escherichia coli*: Cloning and sequencing of the *FPG* structural gene and overproduction of the protein. EMBO J. 6, 3177-3183.

Boiteux, S., OConnor, T. R., Lederer, F., Gouyette, A., and Laval, J. (1990). Homogeneous *Escherichia coli* Fpg protein. A DNA glycosylase which excises imidazole ring-opened purines and nicks DNA at apurinic/apyrimidinic sites. J. Biol. Chem. 265, 3916-3922.

Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992). Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): Excision of purine lesions in DNA produced by ionizing radiation or photosensitization. Biochemistry (USA) 31, 106-110.

Boorstein, R. J., Hilbert, T. P., Cadet, J., Cunningham, R. P., and Teebor, G. W. (1989). UV-induced pyrimidine hydrates in DNA are repaired by bacterial and mammalian DNA glycosylase activities. Biochemistry 28, 6164-6170.

Braithwaite, E., Wu, X. H., and Wang, Z. G. (1999). Repair of DNA lesions: mechanisms and relative repair efficiencies. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 424, 207-219.

Breimer, L. H., and Lindahl, T. (1984). DNA glycosylase activities for thymine residues damaged by ring saturation, fragmentation, or ring contraction are functions of endonuclease III in *Escherichia coli*. J. Biol. Chem. 259, 5543-5548.

Brennan, R. J., Swoboda, B. E. P., and Schiestl, R. H. (1994). Oxidative mutagens induce intrachromosomal recombination in yeast. Mutation Research 308, 159-167.

Bressac, B., Kew, M., Wands, J., and Ozturk, M. (1991). Selective g-mutation to t-mutation of p53 gene in hepatocellular-carcinoma from southern africa. Nature 350, 429-431.

Brookes, P. (1990). The early history of the biological alkylating-agents, 1918-1968. Mutation Research 233, 3-14.

Broomfield, S., Chow, B. L., and Xiao, W. (1998). *MMS2*, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc. Natl. Acad. Sci. USA 95, 5678-5683.

Brown, T.A., (1990). Gene cloning, an introduction. Second edition. Chapman and Hall.

Bruner, S. D., Deng, L., Nash, H. M., Lu, R., Wintner, T. H., and Verdine, G. L. (1997). Chemical biology of DNA repair. FASEB Journal 11(9 Ss), 2551.

Bruner, S. D., Nash, H. M., Lane, W. S., and Verdine, G. L. (1998). Repair of oxidatively damaged guanine in *Saccharomyces cerevisiae* by an alternative pathway. Curr. Biol. 8, 393-403.

Brzobohaty, B., and Kovac, L. (1986). Factors enhancing genetic-transformation of intact yeast-cells modify cell-wall porosity. Journal of General Microbiology 132(Pt11), 3089-3093.

Burns, J. L., Guzder, S. N., Sung, P., Prakash, S., and Prakash, L. (1996). An affinity of human replication protein-A for ultraviolet-damaged DNA - implications for damage recognition in nucleotide excision- repair. J. Biol. Chem. 271, 11607-11610.

Cadet, J., and Berger, M. (1985). Radiation-induced decomposition of the purine-bases within DNA and related model compounds. International Journal of Radiation Biology 47, 127-143.

Cappelli, E., Taylor, R., Cevasco, M., Abbondandolo, A., Caldecott, K., and Frosina, G. (1997). Involvement of XRCC1 and DNA ligase III gene products in DNA base excision repair. J. Biol. Chem. 272, 23970-23975.

Carr, A. M., Sheldrick, K. S., Murray, J. M., AlHarithy, R., Watts, F. Z., and Lehmann, A. R. (1993). Evolutionary conservation of excision repair in *Schizosaccharomyces pombe*: Evidence for a family of sequences related to the *Saccharomyces cerevisiae RAD2* gene. Nucleic Acids Res. 21, 1345-1349.

CassierChauvat, C., and Fabre, F. (1991). A similar defect in UV-induced mutagenesis conferred by the *rad6* and *rad18* mutations of *Saccharomyces cerevisiae*. Mutation Research - DNA Repair 254, 247-253.

Castaing, B., Geiger, A., Seliger, H., Nehls, P., Laval, J., Zelwer, C., and Boiteux, S. (1993). Cleavage and binding of a DNA fragment containing a single 8-oxoguanine by wild type and mutant Fpg proteins. Nucleic Acids Res. 21, 2899-2905.

Cerutti, A. A. (1976). Base damage by ionizing radiation. In 'Photobiology and photochemistry of nucleic acids'. (New York, San Francisco, London: Academic Press).

Chakraborti, T., Ghosh, S. K., Michael, J. R., Batabyal, S. K., and Chakraborti, S. (1998). Targets of oxidative stress in cardiovascular system. Molecular and Cellular Biochemistry 187, 1-10.

Chetsanga, C. J., and Grigorian, C. (1985). In situ enzymatic reclosure of opened imidazole rings of purines in DNA damaged by gamma -irradiation. Proc. Natl. Acad. Sci. USA 82, 633-637.

Chetsanga, C. J., Lozon, M., Makaroff, C., and Savage, L. (1981). Purification and characterization of *Escherichia-coli* formamidopyrimidine-DNA glycosylase that excises damaged 7-methylguanine from deoxyribonucleic-acid. Biochemistry 20, 5201-5207.

Christians, F. C., and Hanawalt, P. C. (1993). Lack of transcription-coupled repair in mammalian ribosomal-RNA genes. Biochemistry 32, 10512-10518.

Clayson, D. B., Mehta, R., and Iverson, F. (1994). Oxidative DNA damage - The effects of certain genotoxic and operationally non-genotoxic carcinogens. Mutation Research - Reviews in Genetic Toxicology 317, 25-42.

Clayton, D. A., Doda, J. N., and Friedberg, E. C. (1974). The absence of pyrimidine dimer repair mechanism in mammalian mitochondria. Proc. Natl. Acad. Sci. USA 71, 2777-2781.

Cleaver, J. E., and Hultner, M. L. (1995). Transcription-related human disorders. American Journal of Human Genetics 56, 1257-1261.

Clever, B., Interthal, H., SchmuckliMaurer, J., King, J., Sigrist, M., and Heyer, W. D. (1997). Recombinational repair in yeast: Functional interactions between Rad51 and Rad54 proteins. EMBO J. 16, 2535-2544.

Cloud, K. G., Shen, B. H., Strniste, G. F., and Park, M. S. (1995). XPG protein has a structure-specific endonuclease activity. Mutation Research Letters 347, 55-60.

Collins, A. R. (1999). Oxidative DNA damage, antioxidants, and cancer. Bioessays 21, 238-246.

Cooper, P. K., Nouspikel, T., Clarkson, S. G., and Leadon, S. A. (1997). Defective transcription-coupled repair of oxidative base damage in Cockayne syndrome patients from XP group G. Science 275, 990-993.

Cunningham, R. P. (1997). DNA glycosylases. Mutation Research-DNA Repair 383, 189-196.

Cunningham, R. P., and Weiss, B. (1985). Endonuclease III (*nth*) mutants of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 82, 474-478.

Cunningham, R. P., Asahara, H., Bank, J. F., Scholes, C. P., Salerno, J. C., Surerus, K., Munck, E., McCracken, J., Peisach, J., and Emptage, M. H. (1989). Endonuclease III is an iron-sulfur protein. Biochemistry (USA) 28, 4450-4455.

Czeczot, H., Tudek, B., Lambert, B., Laval, J., and Boiteux, S. (1991). *Escherichia coli* Fpg protein and UvrABC endonuclease repair DNA damage induced by methylene blue plus visible light *in vivo* and *in vitro*. Journal of Bacteriology 173, 3419-3424.

Dammann, R., and Pfeifer, G. P. (1997). Lack of gene- and strand-specific DNA repair in RNA polymerase III-transcribed human tRNA genes. Mol. Cell. Biol. 17, 219-229.

Dandona, P., Thusu, K., Cook, S., Snyder, B., Makowski, J., Armstrong, D., and Nicotera, T. (1996). Oxidative damage to DNA in diabetes mellitus. Lancet 347, 444-445.

DayaGrosjean, L., Robert, C., Drougard, C., Suarez, H., and Sarasin, A. (1993). High mutation frequency in ras genes of skin tumors isolated from DNA repair deficient xeroderma pigmentosum patients. Cancer Research 53, 1625-1629.

Demple, B., and Harrison, L. (1994). Repair of oxidative damage to DNA: Enzymology and Biology. Annu. Rev. Biochem. 63, 915-948.

de Oliveira, R., Auffret, P., Thoma, D., Geiger, A., Nehls, P., and Boiteux, S. (1994). Fapy DNA gycosylase in the yeast *S. cerevisiae*. Nucleic Acids Res. 22, 3760-3764.

Dianov, G., Bischoff, C., Piotrowski, J., and Bohr, V. A. (1998). Repair pathways for processing of 8-oxoguanine in DNA by mammalian cell extracts. J. Biol. Chem. 273, 33811-33816.

Dizdaroglu, M. (1991). Chemical determination of free radical-induced damage to DNA. Free Radical Biology and Medicine 10, 225-242.

Dizdaroglu, M., Nackerdien, Z., Chao, B. C., Gajewski, E., and Rao, G. (1991a). Chemical nature of *in vivo* DNA base damage in hydrogen peroxide-treated mammalian cells. Archives of Biochemistry and Biophysics 285, 388-390.

Dizdaroglu, M., Rao, G., Halliwell, B., and Gajewski, E. (1991b). Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. Archives of Biochemistry and Biophysics 285, 317-324.

Doetsch, P. W., and Cunningham, R. P. (1990). The enzymology of apurinic apyrimidinic endonucleases. Mutation Research 236, 173-201.

Drapkin, R., and Reinberg, D. (1994). The multifunctional TFIIH complex and transcriptional control. Trends Biochem. Sci. 19, 504-508.

Dujon, B. (1996). The yeast genome project: What did we learn? Trends in Genetics 12, 263-270.

Eide, L., Bjoras, M., Pirovano, H., Alseth, I., Berdal, K. G., and Seeberg, E. (1996). Base excision of oxidative purine and pyrimidine DNA damage in *Saccharomyces cerevisiae* by a DNA glycosylase with sequence similarity to endonuclease III from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 93, 10735-10740.

Eisenbud, M. and Gesell, T. (1998). Environmental Radioactivity from Natural, Industrial and Military Sources. 4th Edition. Academic press, Inc.

Evans, E., Fellows, J., Coffer, A., and Wood, R. D. (1997). Open complex formation around a lesion during nucleotide excision repair provides a structure for cleavage by human XPG protein. EMBO J. 16, 625-638.

Feaver, W. J., Svejstrup, J. Q., Bardwell, L., Bardwell, A. J., Buratowski, S., Gulyas, K. D., Donahue, T. F., Friedberg, E. C., and Kornberg, R. D. (1993). Dual roles of a multiprotein complex from *S-cerevisiae* in transcription and DNA-repair. Cell 75, 1379-1387.

Finkel, E. (1997). Piecing together the puzzle of ageing. Lancet 350, 1150.

Flejter, W. L., McDaniel, L. D., Askari, M., Friedberg, E. C., and Schultz, R. A. (1992a). Characterization of a complex chromosomal rearrangement maps the locus for *in vitro* complementation of xeroderma pigmentosum group D to human chromosome band 19q13. Genes Chromosomes and Cancer 5, 335-342.

Flejter, W. L., McDaniel, L. D., Johns, D., Friedberg, E. C., and Schultz, R. A. (1992b). Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: Involvement of the human *ERCC2* DNA repair gene. Proc. Natl. Acad. Sci. USA 89, 261-265.

Floyd, R. A. (1990a). The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis 11, 1447-1450.

Floyd, R. A. (1990b). Role of oxygen free-radicals in carcinogenesis and brain ischemia. FASEB Journal 4, 2587-2597.

Floyd, R. A. (1990c). The development of a sensitive analysis for 8-hydroxy-2'-deoxyguanosine. Free Radical Research Communications 8, 139-141.

Floyd, R. A., Watson, J. J., Harris, J., West, M., and Wong, P. K. (1986). Formation of 8-hydroxydeoxyguanosine, hydroxyl free-radical adduct of DNA in granulocytes exposed to the tumor promoter, tetradeconylphorbolacetate. Biochemical and Biophysical Research Communications 137, 841-846.

Floyd, R. A., West, M. S., Eneff, K. L., and Schneider, J. E. (1990a). Mediation of 8-hydroxy-guanine formation in DNA by thiazin dyes plus light. Free Radical Biology and Medicine 8, 327-330.

Floyd, R. A., West, M. S., Eneff, K. L., Schneider, J. E., Wong, P. K., Tingey, D. T., and Hogsett, W. E. (1990b). Conditions influencing yield and analysis of 8-hydroxy-2'-deoxyguanosine in oxidatively damaged DNA. Analytical Biochemistry 188, 155-158.

Frankenberg-Schwager, M., and Frankenberg, D. (1990). DNA doublestrand breaks: their repair and relationship to cell killing in yeast. International Journal of Radiation Biology 58, 569-575.

Freese, E. (1959). The specific mutagenic effect of base analogs on phage T4. J. Mol. Biol. 1, 87-105.

Friedberg, E. C. (1985). DNA Repair. (New York: WH Freeman and Company).

Friedberg, E. C. (1988). Deoxyribonucleic acid repair in the yeast *Saccharomyces cerevisiae*. Microbiological Reviews 52, 70-102.

Friedberg, E. C. (1995). Out of the shadows and into the light - the emergence of DNA-repair. Trends Biochem. Sci. 20, 381.

Friedberg, E. C. (1996). Relationships between DNA-repair and transcription. Annu. Rev. Biochem. 65, 15-42.

Friedberg, E. C., Walker, G. C., and Siede, W. (1995). DNA Repair and Mutagenesis. (Washington, DC: American Society of Microbiology Press).

Fritz, L. K., and Smerdon, M. J. (1995). Repair of UV damage in actively transcribed ribosomal genes. Biochemistry 34, 13117-13124.

Game, J. C. (1993). DNA double-strand breaks and the *RAD50-RAD57* genes in saccharomyces. Seminars in Cancer Biology 4, 73-83.

Gietz, D., St. Jean, R. A., and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425-1426.

Girard, P. M., and Boiteux, S. (1997). Repair of oxidized DNA bases in the yeast *Saccharomyces cerevisiae*. Biochimie 79, 559-566.

Girard, P. M., Guibourt, N., and Boiteux, S. (1997). The Ogg1 protein of *Saccharomyces cerevisiae*: A 7,8-dihydro-8-oxoguanine DNA glycosylase AP lyase whose lysine 241 is a critical residue for catalytic activity. Nucleic Acids Res. 25, 3204-3211.

Girard, P. M., DHam, C., Cadet, J., and Boiteux, S. (1998). Opposite base-dependent excision of 7,8-dihydro-8-oxoadenine by the Ogg1 protein of *Saccharomyces cerevisiae*. Carcinogenesis 19, 1299-1305.

Goel, R., and Khanduja, K. L. (1998). Oxidative stress-induced apoptosis - An overview. Current Science 75, 1338-1345.

GonzalezFraguela, M. E., CastellanoBenitez, O., and GonzalezHoyuela, M. (1999). Oxidative stress in neurodegeneration. Revista De Neurologia 28, 504-511.

Goodhead, D. T. (1989). The initial damage produced by ionizing radiations. International Journal of Radiation Biology 56, 623-634.

Goodrich, J. A., and Tjian, R. (1994). Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA-polymerase-II. Cell 77, 145-156.

Graves, R. J., Felzenszwalb, I., Laval, J., and Oconnor, T. R. (1992). Excision of 5'-terminal deoxyribose phosphate from damaged DNA is catalyzed by the Fpg protein of *Escherichia-coli*. J. Biol. Chem. 267, 14429-14435.

Grishko, V. I., Driggers, W. J., LeDoux, S. P., and Wilson, G. L. (1997). Repair of oxidative damage in nuclear DNA sequences with different transcriptional activities. Mutation Research-DNA Repair 384, 73-80.

Grollman, A. P., and Moriya, M. (1993). Mutagenesis by 8-oxoguanine: An enemy within. Trends in Genetics 9, 246-249.

Guschlbauer, W., Duplaa, A. M., Guy, A., Teoule, R., and Fazakerley, G. V. (1991). Structure and in *vitro* replication of DNA templates containing 7,8-dihydro-8-oxoadenine. Nucleic Acids Res. 19, 1753-1758.

Guzder, S. N., Kelley, M. R., and Deutsch, W. A. (1991). Drosophila methyltransferase activity and the repair of alkylated DNA. Mutation Research 255, 143-153.

Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1993). Yeast DNA-repair gene *RAD14* encodes a zinc metalloprotein with affinity for ultraviolet-damaged DNA. Proc. Natl. Acad. Sci. USA 90, 5433-5437.

Guzder, S. N., Qiu, H., Sommers, C. H., Sung, P., Prakash, L., and Prakash, S. (1994a). DNA repair gene *RAD3* of *S. cerevisiae* is essential for transcription by RNA polymerase II. Nature 367, 91-94.

Guzder, S. N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. (1994b). *RAD25* is a DNA helicase required for DNA repair and RNA polymerase II transcription. Nature 369, 578-581.

Guzder, S. N., Bailly, V., Sung, P., Prakash, L., and Prakash, S. (1995a). Yeast DNA-repair protein Rad23 promotes complex-formation between transcription factor TFIIH and DNA-damage recognition factor rad14. J. Biol. Chem. 270, 8385-8388.

Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995b). Reconstitution of Yeast Nucleotide Excision Repair with Purified Rad Proteins, Replication Protein A, and Transcription Factor TFIIH. J. Biol. Chem. 270, 12973-12976.

Guzder, S. N., Sung, P., Prakash, S., and Prakash, L. (1995c). Lethality in yeast of trichothiodystrophy (TTD) mutations in the human xeroderma-pigmentosum group-d gene - implications for transcriptional defect in TTD. J. Biol. Chem. 270, 17660-17663.

Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1996a). *RAD26*, the yeast homolog of human Cockayne's syndrome group B gene, encodes a DNA-dependent ATPase. J. Biol. Chem. 271, 18314-18317.

Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1996b). Nucleotide excision repair in yeast is mediated by sequential assembly of repair factors and not by a pre-assembled repairosome. J. Biol. Chem. 271, 8903-8910.

Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1997). Yeast Rad7-Rad16 complex, specific for the nucleotide excision repair of the nontranscribed DNA strand, is an ATP-dependent DNA damage sensor. J. Biol. Chem. 272, 21665-21668.

Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998a). Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. J. Biol. Chem. 273, 31541-31546.

Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998b). The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition. J. Biol. Chem. 273, 6292-6296.

Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1993). Yeast excision repair gene *RAD2* encodes a single-stranded DNA endonuclease. Nature 366, 365-368.

Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1994a). Human xeroderma pigmentosum group G gene encodes a DNA endonuclease. Nucleic Acids Res. 22, 3312-3316.

Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1994b). Holliday junction cleavage by yeast Rad1 protein. Nature 371, 531-534.

Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1994c). A conserved 5' to 3' exonuclease activity in the yeast and human nucleotide excision-repair proteins Rad2 and XPG. J. Biol. Chem. 269, 31342-31345.

Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995). Structure-specific nuclease activity in yeast nucleotide excision repair protein Rad2. J. Biol. Chem. 270, 30194-30198.

Habraken, Y., Sung, P., Prakash, S., and Prakash, L. (1996). Transcription factor TFIIH and DNA endonuclease Rad2 constitute yeast nucleotide excision repair factor 3: Implications for nucleotide excision repair and Cockayne syndrome. Proc. Natl. Acad. Sci. USA 93, 10718-10722.

Halliwell, B., (1998). Can oxidative DNA damage be used as a biomarker of cancer risk in humans? Problems, resolutions and preliminary results from nutritional supplementation studies. Free radical research, 29,6, 469-486

Hamilton, K. K., Kim, P. M. H., and Doetsch, P. W. (1992). A eukaryotic DNA glycosylase/lyase recognizing ultraviolet light-induced pyrimidine dimers. Nature 356, 725-728.

Hampsey, M. (1991). A tester system for detecting each of the six base-pair substitutions in *Saccharomyces cerevisiae* by selecting for an essential cysteine in iso-1-cytochrome C. Genetics 128, 59-67.

Hanawalt, P. C., Cooper, P. K., Ganesan, A. K., and Smith, C. A. (1979). DNA repair in bacteria and mammalian cells. Annu. Rev. Biochem. 48, 783-836.

Hatahet, Z., Kow, Y. W., Purmal, A. A., Cunningham, R. P., and Wallace, S. S. (1994). New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy- 2'-deoxyuridine are

substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycosylase. J. Biol. Chem. 269, 18814-18820.

Hatch, M. C., Warburton, D., and Santella, R. M. (1990). Polycyclic aromatic hydrocarbon - DNA adducts in spontaneously aborted fetal tissue. Carcinogenesis 11, 1673-1675.

Hayes, R. C., Petrullo, L. A., Huang, H., Wallace, S. S., and Leclerc, J. E. (1988). Oxidative damage in DNA - lack of mutagenicity by thymine glycol lesions. J. Mol. Biol. 201, 239-246.

He, Z., Henricksen, L. A., Wold, M. S., and Ingles, C. J. (1995). RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. Nature 374, 566-569.

Hearst, J. E. (1995). The structure of photolyase - using photon energy for DNA-repair. Science 268, 1858-1859.

Helbock, H. J., Beckman, K. B., Shigenaga, M. K., Walter, P. B., Woodall, A. A., Yeo, H. C., and Ames, B. N. (1998). DNA oxidation matters: The HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc. Natl. Acad. Sci. USA 95, 288-293.

Helbock, H. J., Beckman, K. B., and Ames, B. N. (1999). 8-hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. Methods in Enzymology 300, 156-166.

Henning, K. A., Li, L., Iyer, N., McDaniel, L. D., Reagan, M. S., Legerski, R., Schultz, R. A., Stefanini, M., Lehmann, A. R., Mayne, L. V., and Friedberg, E. C. (1995). The cockayne-syndrome group-a gene encodes a protein that interacts with CSB protein and a subunit of RNA-polymerase-II TFIIH. Cell 82, 555-564.

Hoeijmakers, J. H. J. (1993a). Nucleotide excision repair II: From yeast to mammals. Trends in Genetics 9, 211-217.

Hoeijmakers, J. H. J. (1993b). Nucleotide excision repair I: From *E. coli* to yeast. Trends in Genetics 9, 173-177.

html-1, Sherman-F, 1998; *An Introduction to the Genetics and Molecular Biology of the Yeast Saccharomyces cerevisiae.* http://www.urmc.rochester.edu/smd/biochem/yeast/1.html.

html-2, The Radiation Information Network; Idaho State University; http://www.physics.isu.edu/radinf/index.html.

Husain, I., Griffith, J., and Sancar, A. (1988). Thymine dimers bend DNA. Proc. Natl. Acad. Sci. USA 85, 2558-2562.

Hutchinson, F. (1985). Chemical-changes induced in DNA by ionizing-radiation. Progress in Nucleic Acid Research and Molecular Biology 32, 115-154.

Imlay, J. A., Chin, S. M., and Linn, S. (1988). Toxic DNA damage by hydrogen peroxide through the fenton reaction *in vivo* and *in vitro*. Science 240, 640-642.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14, 1511-1527.

Jansen, L. E. T., Verhage, R. A., and Brouwer, J. A. (1998). Preferential binding of yeast Rad4 -Rad23 complex to damaged DNA. J. Biol. Chem. 273, 33111-33114.

Kamiya, H., Muratakamiya, N., Koizume, S., Inoue, H., Nishimura, S., and Ohtsuka, E. (1995). 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in hot-spots of the C-ha-ras gene - effects of sequence contexts on mutation spectra. Carcinogenesis 16, 883-889.

Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A., and Tanooka, H. (1986). Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. Carcinogenesis 7, 1849-1851.

Kirkwood, T. B. L. (1989). DNA, mutations and aging. Mutation Research 219, 1-7.

Klein, J. C., Bleeker, M. J., Saris, C. P., Roelen, H. C. P. F., Brugghe, H. F., Vandenelst, H., Vandermarel, G. A., Vanboom, J. H., Westra, J. G., Kriek, E., and Berns, A. J. M. (1992). Repair and replication of plasmids with site-specific 8-oxodG and 8-aafdG residues in normal and repair-deficient human-cells. Nucleic Acids Res. 20, 4437-4443.

Klungland, A., Hoss, M., Gunz, D., Constantinou, A., Clarkson, S. G., Doetsch, P. W., Bolton, P. H., Wood, R. D., and Lindahl, T. (1999). Base excision repair of oxidative DNA damage activated by XPG protein. Molecular Cell 3, 33-42.

Kolodner, R. D. (1995). Mismatch repair - mechanisms and relationship to cancer susceptibility. Trends Biochem. Sci. 20, 397-401.

Kolodner, R. (1996). Biochemistry and genetics of eukaryotic mismatch repair. Genes Dev. 10, 1433-1442.

Kolodner, R. D., and Marsischky, G. T. (1999). Eukaryotic DNA mismatch repair. Curr. Opin. Genet. Dev. 9, 89-96.

Kouchakdjian, M., Bodepudi, V., Shibutani, S., Eisenberg, M., Johnson, F., Grollman, A. P., and Patel, D. J. (1991). Nmr structural studies of the ionizing-radiation adduct 7-hydro-8-oxodeoxyguanosine (8-oxo-7H-dG) opposite deoxyadenosine in a DNA duplex - 8-oxo-7H-dG(syn).dA(anti) alignment at lesion site. Biochemistry 30, 1403-1412.

Kow, Y. W. (1989). Mechanism of action of *Escherichia coli* exonuclease III. Biochemistry (USA) 28, 3280-3287.

Kow, Y. W. (1994). Base excision repair in *E. coli* - An overview. Annals of the New York Academy of Sciences 726, 178-180.

Kow, Y. W., Wallace, S. S., and vanHouten, B. (1990). UvrABC nuclease complex repairs thymine glycol, an oxidative DNA base damage. Mutation Research - DNA Repair 235, 147-156.

Kramer, W., Kramer, B., Williamson, M. S., and Fogel, S. (1989). Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae*: Homology of PMS1 to procaryotic MutL and HexB. Journal of Bacteriology 171, 5339-5346.

Krokan, H. E., Standal, R., and Slupphaug, G. (1997). DNA glycosylases in the base excision repair of DNA. Biochemical Journal 325(Pt1), 1-16.

Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., and Nishimura, S. (1987). Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. Nature 327, 77-79.

Kunala, S., and Brash, D. E. (1995). Intragenic domains of strand-specific repair in *Escherichia-coli*. J. Mol. Biol. 246, 264-272.

Kung, H. C., and Bolton, P. H. (1997). Structure of a duplex DNA containing a thymine glycol residue in solution. J. Biol. Chem. 272, 9227-9236.

Kunz, B. A., Xiaolin Kang, and Kohalmi, L. (1991). The yeast *rad18* mutator specifically increases GC-TA transversions without reducing correction of GA or CT mispairs to GC. Mol. Cell. Biol. 11, 218-225.

Kurjan, J. (1993). The pheromone response pathway in *S-cerevisiae*. Annu. Rev. Genet. 27, 147-179.

Lamola, A.A, (1969). Specific formation of thymine dimers in DNA. Photochem.Photobiol. 9, 291-294.

Lamola, A. (1974). Functional aspects of the spectroscopy and photochemistry of organic compounds; electric energy transfer in biologic systems and photosensitization. P. 17-55. *In* M.A. Pathak, L.C.Harber, M. Seij, and A. Kutika (ed), *Sun light and Man*. University of Tokyo press, Tokyo.

Laval, J. (1996). Role of DNA repair enzymes in the cellular resistance to oxidative stress. Pathologie Biologie 44, 14-24.

Laval, J., Boiteux, S., and OConnor, T. R. (1990). Physiological properties and repair of apurinic/apyrimidinic sites and imidazole ring-opened guanines in DNA. Mutation Research 233, 73-79.

Leadon, S. A., and Avrutskaya, A. V. (1998). Requirement for DNA mismatch repair proteins in the transcription-coupled repair of thymine glycols in *Saccharomyces cerevisiae*. Mutation Research-DNA Repair 407, 177-187.

Leadon, S. A., and Cooper, P. K. (1993). Preferential repair of ionizing radiation-induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome. Proc. Natl. Acad. Sci. USA 90, 10499-10503.

Leadon, S. A., and Lawrence, D. A. (1992). Strand-selective repair of DNA damage in the yeast *GAL7* gene requires RNA polymerase II. J. Biol. Chem. 267, 23175-23182.

Leadon, S. A., Barbee, S. L., and Dunn, A. B. (1995). The yeast *RAD2*, but not *RAD1*, gene is involved in the transcription-coupled repair of thymine glycols. Mutation Research-DNA Repair 337, 169-178.

Lehmann, A. R. (1989). Trichothiodystrophy and the relationship between DNA-repair and cancer. Bioessays 11, 168-170.

Lehmann, A. R. (1998). Dual functions of DNA repair genes: molecular, cellular, and clinical implications. Bioessays 20, 146-155.

Lehmann, A. R., and Norris, P. G. (1989). DNA repair and cancer: Speculations based on studies with xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy. Carcinogenesis 10, 1353-1356.

Lehmann, A. R., Bridges, B. A., Hanawalt, P. C., Johnson, R. T., Kanaar, R., Krokan, H. E., Kyrtopoulos, S., Lambert, B., Melton, D. W., Moustacchi, E., Natarajan, A. T., Radman, M., Sarasin, A., Seeberg, E., Smerdon, M. J., Smith, C. A., Smith, P. J., Thacker, J., Thomale, J., Waters, R., Weeda, G., West, S. C., vanZeeland, A. A., and Zdzienicka, M. Z. (1996). Workshop on processing of DNA damage. Mutation Research-DNA Repair 364, 245-270.

Lenaz, G. (1998). Role of mitochondria in oxidative stress and ageing. Biochimica Et Biophysica Acta-Bioenergetics 1366, 53-67.

Li, L., Lu, X. Y., Peterson, C. A., and Legerski, R. J. (1995). An interaction between the DNA-repair factor XPA and replication protein-a appears essential for nucleotide excision-repair. Mol. Cell. Biol. 15, 5396-5402.

Li, S., and Waters, R. (1996). Nucleotide level detection of cyclobutane pyrimidine dimers using oligonucleotides and magnetic beads to facilitate labelling of DNA fragments incised at the dimers and chemical sequencing reference ladders. Carcinogenesis 17, 1549-1552.

Lin, J. J., and Sancar, A. (1989). A new mechanism for repairing oxidative damage to DNA - (A)BC excinuclease removes AP sites and thymine glycols from DNA. Biochemistry 28, 7979-7984.

Lin, J. J., and Sancar, A. (1992). Excinuclease - the *Escherichia-coli* nucleotide excision repair enzyme. Molecular Microbiology 6, 2219-2224.

Lin, J. J., Phillips, A. M., Hearst, J. E., and Sancar, A. (1992). Active-site of (a)bc excinuclease .2. Binding, bending, and catalysis mutants of *uvrb* reveal a direct role in 3' and an indirect role in 5' incision. J. Biol. Chem. 267, 17693-17700.

Lindahl, T. (1979). DNA glycosylases, endonucleases for apurinic/apyrididinic sites and base excision repair. Progress in Nucleic Acid Research and Molecular Biology 22, 135-192.

Lindahl, T., Karran, P., and Wood, R. D. (1997). DNA excision repair pathways. Curr. Opin. Genet. Dev. 7, 158-169.

Ljungman, M., and Hanawalt, P. C. (1992a). Efficient protection against oxidative DNA damage in chromatin. Molecular Carcinogenesis 5, 264-269.

Loveless, A. (1966). Genetic and allied effects of alkylating agents. Butterworth, London.

Maki, H., and Sekiguchi, M. (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355, 273-275.

Mallet, L., Bussereau, F., and Jacquet, M. (1995). A 43.5-Kb segment of yeast chromosome-XIV, which contains *MFA2, MEP2, CAP/SRV2, NAM9, FKB1/FPR1/RBP1, MOM22* and *CPT1*, predicts an adenosine-deaminase gene and 14 new open reading frames. Yeast 11, 1195-1209.

Marsischky, G. T., Filosi, N., Kane, M. F., and Kolodner, R. D. (1996). Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. Genes Dev. 10, 407-420.

Matsunaga, T., Park, C., -H., Bessho, T., Mu, D., and Sancar, A. (1996). Replication factor A confers structure specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA repair excision nuclease. J. Biol. Chem. 271, 11047-11050.

Mazumder, A., Gerlt, J. A., Absalon, M. A., Stubb, J. A., Cunningham, R. P., Withka, J., and Bolton, P. H. (1991). Stereochemical studies of the beta-elimination reactions at aldehyde abasic sites in DNA: endonuclease III from *E. coli*, sodium hydroxide, and Lys-Trp-Lys. Biochemistry 30, 1119-1126.

McAuleyhecht, K. E., Leonard, G. A., Gibson, N. J., Thomson, J. B., Watson, W. P., Hunter, W. N., and Brown, T. (1994). Crystal-structure of a DNA duplex containing 8-hydroxydeoxyguanine-adenine base-pairs. Biochemistry 33, 10266-10270.

McBride, T. J., Preston, B. D., and Loeb, L. A. (1991). Mutagenic spectrum resulting from DNA damage by oxygen radicals. Biochemistry 30, 207-213.

McBride, T. J., Schneider, J. E., Floyd, R. A., and Loeb, L. A. (1992). Mutations induced by methylene blue plus light in single-stranded M13mp2. Proc. Natl. Acad. Sci. USA 89, 6866-6870.

McCready, S. (1994). Repair of 6-4 photoproducts and cyclobutane pyrimidine dimers in *rad* mutants of *S.cerevisiae*. Mutation Research-DNA Repair 315, 261-273.

McDonald, J. P., Levine, A. S., and Woodgate, R. (1997). The *Saccharomyces cerevisiae RAD30* gene, a homologue of *Escherichia coli dinB* and *umuC*, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. Genetics 147, 1557-1568.

McGoldrick, J. P., Yeh, Y. C., Solomon, M., Essigmann, J. M., and Lu, A. L. (1995). Characterisation of a mammalian homolog of the Esherichia coli MutY mismatch repair protein. Mol. Cell. Biol. 15, 989-996.

Melamede, R. J., Hatahet, Z., Kow, Y. W., Ide, H., and Wallace, S. S. (1994). Isolation and characterization of endonuclease-VIII from escherichia- coli. Biochemistry 33, 1255-1264.

Mellon, I., and Hanawalt, P. C. (1989). Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand. Nature 342, 95-98.

Mellon, I., Spivak, G., and Hanawalt, P. C. (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian *DHFR* gene. Cell 51, 241-249.

Meniel, V., and Waters, R. (1999). Spontaneous and photosensitiser-induced DNA single-strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites at nucleotide resolution in the nuclear and mitochondrial DNA of *Saccharomyces cerevisiae*. Nucleic Acids Res. 27, 822-830.

Michaels, M. L., and Miller, J. H. (1992). The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). Journal of Bacteriology 174, 6321-6325.

Michaels, M. L., Pham, L., Nghiem, Y., Cruz, C., and Miller, J. H. (1990). MutY, an adenine glycosylase active on G-A mispairs, has homology to endonuclease III. Nucleic Acids Res. 18, 3841-3845.

Michaels, M. L., Pham, L., Cruz, C., and Miller, J. H. (1991). MutM, a protein that prevents GC to TA transversions, is formamidopyrimidine-DNA glycosylase. Nucleic Acids Res. 19, 3629-3632.

Michaels, M. L., Cruz, C., Grollman, A. P., and Miller, J. H. (1992). Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc. Natl. Acad. Sci. USA 89, 7022-7025.

Mitchell, D. L., and Nairn, R. S. (1989). The biology of the (6-4) photoproduct. Photochemistry and Photobiology 49, 805-819.

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. 25, 229-253.

Modrich, P., and Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu. Rev. Biochem. 65, 101-133.

Mol, C. D., Arvai, A. S., Sanderson, R. J., Slupphaug, G., Kavli, B., Krokan, H. E., Mosbaugh, D. W., and Tainer, J. A. (1995a). Crystal-structure of human uracil-DNA glycosylase in complex with a protein inhibitor - protein mimicry of DNA. Cell 82, 701-708.

Mol, C. D., Arvai, A. S., Slupphaug, G., Kavli, B., Alseth, I., Krokan, H. E., and Tainer, J. A. (1995b). Crystal-structure and mutational analysis of human uracil-DNA glycosylase - structural basis for specificity and catalysis. Cell 80, 869-878.

Mol, C. D., Kuo, C. F., Thayer, M. M., Cunningham, R. P., and Tainer, J. A. (1995c). Structure and function of the multifunctional DNA-repair enzyme exonuclease-III. Nature 374, 381-386.

Moller, P., and Wallin, H. (1998). Adduct formation, mutagenesis and nucleotide excision repair of DNA damage produced by reactive oxygen species and lipid peroxidation product. Mutation Research-Reviews in Mutation Research 410, 271-290.

Moriya, M., Ou, C., Bodepudi, V., Johnson, F., Takeshita, M., and Grollman, A. P. (1991). Site-specific mutagenesis using a gapped duplex vector: a study of translesion synthesis past 8-oxodeoxyguanosine in *E.coli*. Mutation Research 254, 281-288.

Mu, D., Park, C. H., Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. (1995). Reconstitution of human DNA-repair excision nuclease in a highly defined system. J. Biol. Chem. 270, 2415-2418.

Mu, D., Hsu, D. S., and Sancar, A. (1996). Reaction-mechanism of human DNA-repair excision nuclease. J. Biol. Chem. 271, 8285-8294.

Mullenders, L. H. F. (1998). Transcription response and nucleotide excision repair. Mutation Research-DNA Repair 409, 59-64.

Muller, E., Boiteux, S., Cunningham, R. P., and Epe, B. (1990). Enzymatic recognition of DNA modifications induced by singlet oxygen and photosensitizers. Nucleic Acids Res. 18, 5969-5973.

Nakai, S., and Matsumoto, S. (1967). Two types of radiation sensitive mutants in yeast. Mutation Research - DNA Repair 4, 129-136.

Nance, M. A., and Berry, S. A. (1992). Cockayne Syndrome: Review of 140 cases. American Journal of Medical Genetics 42, 68-84.

Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S., and Tannenbaum, S. R. (1992). DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. Proc. Natl. Acad. Sci. USA 89, 3030-3034.

Nouspikel, T., Lalle, P., Leadon, S. A., Cooper, P. K., and Clarkson, S. G. (1997). A common mutational pattern in Cockayne syndrome patients from xeroderma pigmentosum group G: Implications for a second XPG function. Proc. Natl. Acad. Sci. USA 94, 3116-3121.

Oconnor, T. R., and Laval, J. (1989). Physical association of the 2,6-diamino-4-hydroxy-5n-formamidopyrimidine-DNA glycosylase of *Escherichia-coli* and an activity nicking DNA at apurinic apyrimidinic sites. Proc. Natl. Acad. Sci. USA 86, 5222-5226.

OConnor, T. R., Boiteux, S., and Laval, J. (1988). Ring-opened 7-methylguanine residues in DNA are a block to *in vitro* DNA synthesis. Nucleic Acids Res. 16, 5879-5894.

Oconnor, T. R., Graves, R. J., Demurcia, G., Castaing, B., and Laval, J. (1993). Fpg protein of *Escherichia-coli* is a zinc finger protein whose cysteine residues have a structural and or functional-role. J. Biol. Chem. 268, 9063-9070.

Oda, Y., Uesugi, S., Ikehara, M., Nishimura, S., Kawase, Y., Ishikawa, H., Inoue, H., and Ohtsuka, E. (1991). NMR-studies of a DNA containing 8-hydroxydeoxyguanosine. Nucleic Acids Res. 19, 1407-1412.

ODonovan, A., Davies, A. A., Moggs, J. G., West, S. C., and Wood, R. D. (1994a). XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. Nature 371, 432-435.

Odonovan, A., Scherly, D., Clarkson, S. G., and Wood, R. D. (1994b). Isolation of active recombinant XPG protein, a human DNA-repair endonuclease. J. Biol. Chem. 269, 15965-15968.

Ohuigin, C., McConnell, D. J., Kelly, J. M., and Vanderputten, W. J. M. (1987). Methylene-blue photosensitized strand cleavage of DNA - effects of dye binding and oxygen. Nucleic Acids Res. 15, 7411-7427.

Orren, D. K., and Sancar, A. (1989). The (A)BC excinuclease of *Escherichia-coli* has only the UvrB and UvrC subunits in the incision complex. Proc. Natl. Acad. Sci. USA 86, 5237-5241.

Orren, D. K., Selby, C. P., Hearst, J. E., and Sancar, A. (1992). Post-incision steps of nucleotide excision repair in *Escherichia coli*. J. Biol. Chem. 267, 780-788.

Papadopoulos, N., Nicholaides, N. C., Wei, Y.-F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Hamilton, S. R., Petersen, G. M., Watson, P., Lynch, H. T., Peltomaki, P., Mecklin, J.-P., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. (1994). Mutation of a mutL homolog in hereditary colon cancer. Science 263, 1625-1629.

Park, C. H., and Sancar, A. (1994). Formation of a ternary complex by human XPA, ERCC1, and ERCC4(XPF) excision-repair proteins. Proc. Natl. Acad. Sci. USA 91, 5017-5021.

Park, E., Guzder, S. N., Koken, M. H. M., JaspersDekker, I., Weeda, G., Hoeijmakers, J. H. J., Prakash, S., and Prakash, L. (1992). *RAD25* (*SSL2*), the yeast homolog of the human xeroderma pigmentosum group B DNA repair gene, is essential for viability. Proc. Natl. Acad. Sci. USA 89, 11416-11420.

Pearlman, D. A., Holbrook, S. R., Pirkle, D. H., and Kim, S.-H. (1985). Molecular models for DNA damaged by photoreaction. Science 227, 1304-1308.

Petit, C., and Sancar, A. (1999). Nucleotide excision repair: From *E-coli* to man. Biochimie 81, 15-25.

Pfeifer, G. P., Drouin, R., Riggs, A. D., and Holmquist, G. P. (1991). *In vivo* mapping of a DNA adduct at nucleotide resolution: Detection of pyrimidine (6-4) pyrimidone photoproducts by ligation mediated polymerase chain reaction. Proc. Natl. Acad. Sci. USA 88, 1374-1378.

Pflaum, M., Will, O., and Epe, B. (1997). Determination of steady-state levels of oxidative DNA base modifications in mammalian cells by means of repair endonucleases. Carcinogenesis 18, 2225-2231.

Pierce, J. R., Case, R., and Tang, M. S. (1989). Recognition and repair of 2-aminofluorene-DNA and 2-(acetylamino)fluorene DNA adducts by UvrABC nuclease. Biochemistry 28, 5821-5826.

Piette, J., Decuyper, J., Mervillelouis, M. P., and Vandevorst, A. (1986a). Biomolecular photoalterations mediated by phenothiazine-derivatives. Biochimie 68, 835-842.

Piette, J., Decuyper, M. P., Decuyper, M. L., and Decuyper, J. (1986b). Damages induced in nucleic-acids by photosensitization. Photochemistry and Photobiology 44, 793-802.

Pinto, A. L., and Lippard, S. J. (1985). Binding of the antitumor drug cis-diamminedichloroplatinum (II) (cisplatin) to DNA. Biochimica et Biophysica Acta 780, 167-180.

Poulsen, H. E., Loft, S., Prieme, H., Vistisen, K., Lykkesfeldt, J., Nyyssonen, K., and Salonen, J. T. (1998). Oxidative DNA damage *in vivo*: Relationship to age, plasma antioxidants, drug metabolism, glutathione-S-transferase activity and urinary creatinine excretion. Free Radical Research 29, 565-571.

Prakash, S., Sung, P., and Prakash, L. (1993). DNA repair genes and proteins of *Saccharomyces cerevisiae*. Annu. Rev. Genet. 27, 33-70.

Prolla, T. A., Christie, D.-M., and Liskay, R. M. (1994a). A requirement in yeast DNA mismatch repair for *MLH1* and *PMS1*, two homologs of the bacterial *mutL* gene. Mol. Cell. Biol. 14, 407-415.

Prolla, T. A., Pang, Q., Alani, E., Kolodner, R. D., and Liskay, R. M. (1994b). Interactions between the MSH2, MLH1 and PMS1 proteins during the initiation of DNA mismatch repair. Science 265, 1091-1093.

Purmal, A. A., Lampman, G. W., Bond, J. P., Hatahet, Z., and Wallace, S. S. (1998). Enzymatic processing of uracil glycol, a major oxidative product of DNA cytosine. J. Biol. Chem. 273, 10026-10035.

Radicella, J. P., Dherin, C., Desmaze, C., Fox, M. S., and Boiteux, S. (1997). Cloning and characterisation of *hOGG1*, a human homolog of the *OGG1* gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 94, 8010-8015.

Radman, M. (1976). An endonuclease from *E. coli* that introduces single polynucleotide chain scissions in UV irradiated DNA. J. Biol. Chem. 251, 1438-1445.

Reardon, J. T., Bessho, T., Kung, H. C., Bolton, P. H., and Sancar, A. (1997). *In vitro* repair of oxidative DNA damage by human nucleotide excision repair system: Possible explanation for neurodegeneration in Xeroderma pigmentosum patients. Proc. Natl. Acad. Sci. USA 94, 9463-9468.

Reed, S. H., Boiteux, S., and Waters, R. (1996a). UV-induced endonuclease III sensitive sites at the mating type loci in *Saccharomyces cerevisiae* are repaired by nucleotide excision repair - *RAD7* and *RAD16* are not required for their removal from HML-alpha. Mol. Gen. Genet. 250, 505-514.

Reed, S. H., McCready, S., Boiteux, S., and Waters, R. (1996b). The repair of endonuclease III sensitive sites, 6-4 photoproducts and cyclobutane pyrimidine dimers is different in a point mutant of *RAD14*, the *S. cerevisiae* homologue of the human gene defective in XPA patients. Mol. Gen. Genet. 250, 515-522.

Richter, C. (1992). Reactive oxygen and DNA damage in mitochondria. Mutation Research - DNAging Genetic Instability and Aging 275, 249-255.

Richter, C., Park, J. W., and Ames, B. N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc. Natl. Acad. Sci. USA 85, 6465-6467.

Riley, P. A. (1994). Free radicals in biology:oxidative stress and the effects of ionizing radiation. International Journal of Radiation Biology 65, 27-33.

Rodriguez, H., Drouin, R., Holmquist, G. P., Oconnor, T. R., Boiteux, S., Laval, J., Doroshow, J. H., and Akman, S. A. (1995). Mapping of copper hydrogen peroxide-induced DNA-damage at nucleotide resolution in human genomic DNA by ligation-mediated polymerase chain-reaction. J. Biol. Chem. 270, 17633-17640.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning. A laboratory manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Samson, L. (1992). The suicidal DNA repair methyltransferases of microbes. Molecular Microbiology 6, 825-831.

Sancar, A. (1996). DNA excision repair. Annu. Rev. Biochem. 65, 43-81.

Sancar, A., and Sancar, G. B. (1988). DNA-repair enzymes. Annu. Rev. Biochem. 57, 29-67.

Sancar, A., and Tang, M. S. (1993). Nucleotide excision repair. Photochemistry and Photobiology 57, 905-921.

Sancar, G. B., and Smith, F. W. (1989). Interactions between yeast photolyase and nucleotide excision repair proteins in *Saccharomyces cerevisiae* and *Escherichia coli*. Mol. Cell. Biol. 9, 4767-4776.

Sandigursky, M., Yacoub, A., Kelley, M. R., Xu, Y., Franklin, W. A., and Deutsch, W. A. (1997). The yeast 8-oxoguanine DNA glycosylase (Ogg1) contains a DNA deoxyribophosphodiesterase (dRpase) activity. Nucleic Acids Res. 25, 4557-4561.

Savva, R., Mcauleyhecht, K., Brown, T., and Pearl, L. (1995). The structural basis of specific base-excision repair by uracil DNA glycosylase. Nature 373, 487-493.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H. J., Chambon, P., and Egly, J. M. (1993). DNA repair helicase: A component of BTF2 (TFIIH) basic transcription factor. Science 259, 58-63.

Scherly, D., Nouspikel, T., Corlet, J., Ucla, C., Bairoch, A., and Clarkson, S. G. (1993). Complementation of the DNA-repair defect in xeroderma-pigmentosum group-g cells by a human cDNA related to yeast RAD2. Nature 363, 182-185.

Schiestl, R. H., and Gietz, R. D. (1989). High-efficiency transformation of intact yeast-cells using single stranded nucleic-acids as a carrier. Current Genetics 16, 339-346.

Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from *S.cerevisiae*. Nucleic Acids Res. 18, 3091-3092.

Schneider, J. E., Price, S., Maidt, L., Gutteridge, J. M. C., and Floyd, R. A. (1990). Methylene-blue plus light mediates 8-hydroxy 2'-deoxyguanosine formation in DNA preferentially over strand breakage. Nucleic Acids Res. 18, 631-635.

Scott, A. D., Neishabury, M., Jones, D. H., Reed, S. H., Boiteux, S., and Waters, R. (1999). Spontaneous mutation, oxidative DNA damage, and the roles of base and nucleotide excision repair in the yeast *Saccharomyces cerevisiae*. Yeast 15, 205-218.

Selby, C. P., and Sancar, A. (1990). Transcription preferentially inhibits nucleotide excision repair of the template DNA strand in *vitro*. J. Biol. Chem. 265, 21330-21336.

Selby, C. P., and Sancar, A. (1991). Gene- and strand-specific repair *in vitro*: Partial purification of a transcription-repair coupling factor. Proc. Natl. Acad. Sci. USA 88, 8232-8236.

Selby, C. P., and Sancar, A. (1993). Transcription-repair coupling and mutation frequency decline. Journal of Bacteriology 175, 7509-7514.

Selby, C. P., and Sancar, A. (1995). Structure and function of transcription-repair coupling factor. II. Catalytic properties. J. Biol. Chem. 270, 4890-4895.

Selby, C. P., Witkin, E. M., and Sancar, A. (1991). *Escherichia-coli mfd* mutant deficient in mutation frequency decline lacks strand-specific repair - *in vitro* complementation with purified coupling factor. Proc. Natl. Acad. Sci. USA 88, 11574-11578.

Setlow, R. B., and Carrier, W. L. (1966). Pyrimidine dimers in UV irradiated DNA's. J. Mol. Biol. 17, 237-254.

Shibutani, S., Takeshita, M., and Grollman, A. P. (1991). Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 349, 431-434.

Shibutani, S., Bodepudi, V., Johnson, F., and Grollman, A. P. (1993). Translesional synthesis on DNA templates containing 8-oxo-7,8-dihydrodeoxyadenosine. Biochemistry 32, 4615-4621.

Shigenaga, M. K., and Ames, B. N. (1991). Assays for 8-hydroxy-2'-deoxyguanosine: A biomarker of *in vivo* oxidative DNA damage. Free Radical Biology and Medicine 10, 211-216.

Shinohara, A., and Ogawa, T. (1995). Homologous recombination and the roles of double-strand breaks. Trends Biochem. Sci. 20, 387-391.

Shiomi, T., Harada, Y. N., Saito, T., Shiomi, N., Okuno, Y., and Yamaizumi, M. (1994). An ERCC5 gene with homology to yeast *RAD2* is involved in group G xeroderma pigmentosum. Mutation Research - DNA Repair 314, 167-175.

Sies, H., and Menck, C. F. M. (1992). Singlet oxygen induced DNA damage. Mutation Research - DNAging Genetic Instability and Aging 275, 367-375.

Sies, H., Stahl, W., and Sundquist, A. R. (1992). Antioxidant functions of vitamins: Vitamins E and C, beta-carotene, and other carotenoids. Annals of the New York Academy of Sciences 669, 7-20.

Singer, B., and Kusmierek, J. T. (1982). Chemical mutagenesis. Annu. Rev. Biochem. 51, 655-693.

Sobol, R. W., Horton, J. K., Kuhn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996). Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature 379, 183-186.

Sun, A. Y., and Chen, Y. M. (1998). Oxidative stress and neurodegenerative disorders. Journal of Biomedical Science 5, 401-414.

Sun, J., Chen, Y., Li, M. T., and Ge, Z. L. (1998). Role of antioxidant enzymes on ionizing radiation resistance. Free Radical Biology and Medicine 24, 586-593.

Sung, P., Prakash, L., Weber, S., and Prakash, S. (1987). The *RAD3* gene of *S. cerevisiae* encodes a DNA dependent ATPase. Proc. Natl. Acad. Sci. USA 84, 6045-6049.

Sung, P., Bailly, V., Weber, C., Thompson, L. H., Prakash, L., and Prakash, S. (1993a). Human xeroderma pigmentosum group D gene encodes a DNA helicase. Nature 365, 852-855.

Sung, P., Reynolds, P., Prakash, L., and Prakash, S. (1993b). Purification and characterization of the *Saccharomyces cerevisiae* Rad1/Rad10 endonuclease. J. Biol. Chem. 268, 26391-26399.

Sung, P., Watkins, J. F., Prakash, L., and Prakash, S. (1994). Negative superhelicity promotes ATP-dependent binding of yeast Rad3 protein to ultraviolet-damaged DNA. J. Biol. Chem. 269, 8303-8308.

Sung, P., Guzder, S. N., Prakash, L., and Prakash, S. (1996). Reconstitution of TFIIH and requirement of its helicase subunits, Rad3 and Rad25, in the incision step of nucleotide excision repair. J. Biol. Chem. 271, 10821-10826.

Svejstrup, J. Q., Wang, Z. G., Feaver, W. J., Wu, X. H., Bushnell, D. A., Donahue, T. F., Friedberg, E. C., and Kornberg, R. D. (1995). Different forms of TFIIH for transcription and DNA-repair - holo- TFIIH and a nucleotide excision repairosome. Cell 80, 21-28.

Swanson, R. L., Morey, N. J., Doetsch, P. W., and JinksRobertson, S. (1999). Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 19, 2929-2935.

Sweder, K. S., and Hanawalt, P. C. (1993). Transcription-coupled DNA-repair. Science 262, 439.

Sweder, K. S., and Hanawalt, P. C. (1994). 2 transcription factors involved in DNA-repair in *S.cerevisiae*. Journal of Cellular Biochemistry, 24.

Sweder, K. S., Chun, R., Mori, T., and Hanawalt, P. C. (1996). DNA-repair deficiencies associated with mutations in genes encoding subunits of transcription initiation-factor TFIIH in yeast. Nucleic Acids Res. 24, 1540-1546.

Szostak, J. W., Orrweaver, T. L., and Rothstein, R. J. (1983). The double-strand-break repair model for recombination. Cell 33, 25-35.

Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. (1989). P53 - a frequent target for genetic abnormalities in lung-cancer. Science 246, 491-494.

Taylor, J.-S., Garrett, D. S., and Cohrs, M. P. (1988). Solution state structure of the Dewar pyrimidinone photoproduct of thymidylyl-(3'-5')-thymidine. Biochemistry 27, 7206-7215.

Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P., and Nishimura, S. (1991). 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. Proc. Natl. Acad. Sci. USA 88, 4690-4694.

Tchou, J., Bodepudi, V., Shibutani, S., Antoshechkin, I., Miller, J., Grollman, A. P., and Johnson, F. (1994). Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA. J. Biol. Chem. 269, 15318-15324.

Teng, Y., Li, S., Waters, R., and Reed, S. H. (1997). Excision repair at the level of the nucleotide in the *Saccharomyces cerevisiae MFA2* gene: the mapping of where enhanced repair in the transcribed strand begins or ends and the identification of only a partial Rad16 requisite for repairing upstream control sequences. J. Mol. Biol. 267, 324-337.

Teng, Y. M., Longhese, M., McDonough, G., and Waters, R. (1998). Mutants with changes in different domains of yeast replication protein a exhibit differences in repairing the control region, the transcribed strand and the non-transcribed strand of the *Saccharomyces cerevisiae MFA2* gene. J. Mol. Biol. 280, 355-363.

Terleth, C., van Sluis, C., and van de Putte, P. (1989). Differential repair of UV damage in *S. cerevisiae*. Nucleic Acids Res. 17, 4433-4439.

Terleth, C., Schenk, P. W., Vansluis, C. A., and Vandeputte, P. (1990). Differential repair of the *MAT* locus of *S.cerevisiae*. Mutagenesis 5, 78.

Terleth, C., van Klaveren, J., Poot, R., van de Putte, P., and Brouwer, J. (1991). Differential repair of UV damage in rad mutants of *Saccharomyces cerevisiae*: the relative influence of transcription and chromatin structure on the repair of resp. *MAT* and *HML* [Ph.D. Dissertation]. (Leiden University).

Thayer, M. M., Ahern, H., Xing, D. X., Cunningham, R. P., and Tainer, J. A. (1995a). Novel DNA-binding motifs in the DNA-repair enzyme endonuclease-III crystal-structure. EMBO J. 14, 4108-4120.

Thayer, M. M., Mol, C. D., Tainer, J. A., Ahern, H., Xing, D. X., and Cunningham, R. P. (1995b). Enzymes for base excision-repair - structural and mechanistic considerations. Journal of Cellular Biochemistry(S21A), 268.

Thomas, D., Scott, A. D., Barbey, R., Padula, M., and Boiteux, S. (1997). Inactivation of *OGG1* increases the incidence of GC to TA transversions in *Saccharomyces cerevisiae*: Evidence for endogenous oxidative damage to DNA in eukaryotic cells. Mol. Gen. Genet. 254, 171-178.

Tijsterman, M., Verhage, R. A., vandePutte, P., TasserondeJong, J. G., and Brouwer, J. (1997). Transitions in the coupling of transcription and nucleotide excision repair within RNA polymerase II-transcribed genes of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 94, 8027-8032.

Tomkinson, A. E., Bardwell, A. J., Bardwell, L., Tappe, N. J., and Friedberg, E. C. (1993). Yeast DNA repair and recombination proteins Rad1 and Rad10 constitute a single-strand-DNA endonuclease. Nature 362, 860-862.

Tretyakova, N. Y., Niles, J. C., Burney, S., Wishnok, J. S., and Tannenbaum, S. R. (1999). Peroxynitrite-induced reactions of synthetic oligonucleotides containing 8-oxoguanine. Chemical Research in Toxicology 12, 459-466.

Troelstra, C., Van Gool, A., De Wit, J., Vermeulen, W., Bootsma, D., and Hoeijmakers, J. H. J. (1992). ERCC6, a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes. Cell 71, 939-953.

Tsai-Wu, J. J., Liu, H. F., and Lu, A. L. (1992). *Escherichia coli* MutY protein has both N-glycosylase and apurinic/apyrimidinic endonuclease activities on A-C and A-G mispairs. Proc. Natl. Acad. Sci. USA 89, 8779-8783.

Tu, Y., Tornaletti, S., and Pfeifer, G. P. (1996). DNA repair domains within a human gene: selective repair of sequences near the transcription initiation site. EMBO J. 15, 675-683.

Tudek, B., Boiteux, S., Laval, J. (1992). Biological properties of imidazole ring-opened N7-methylguanine in M13 mp18 phage DNA . Nucleic Acid Research. 20, 3079-3084.

Umlas, M. E., Franklin, W. A., Chan, G. L., and Haseltine, W. A. (1985). Ultraviolet-light irradiation of defined-sequence DNA under conditions of chemical photosensitization. Photochemistry and Photobiology 42, 265-273.

vanderKemp, P. A., Thomas, D., Barbey, R., deOliveira, R., and Boiteux, S. (1996). Cloning and expression in *Escherichia coli* of the *OGG1* gene of *Saccharomyces cerevisiae*, which codes for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine and 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine. Proc. Natl. Acad. Sci. USA 93, 5197-5202.

van Gool, A. J., Verhage, R., Swagemakers, S. M. A., van de Putte, P., Brouwer, J., Troelstra, C., Bootsma, D., and Hoeijmakers, J. H. J. (1994). *RAD26*, the functional *S.cerevisiae* homolog of the Cockayne syndrome B gene ERRC6. EMBO J. 13, 5361-5369.

vanGool, A. J., vanderHorst, G., Citterio, E., and Hoeijmakers, J. H. J. (1997). Cockayne syndrome: defective repair of transcription? EMBO J. 16, 4155-4162.

Vanhoffen, A., Natarajan, A. T., Mayne, L. V., Vanzeeland, A. A., Mullenders, L. H. F., and Venema, J. (1993). Deficient repair of the transcribed strand of active genes in cockaynes-syndrome cells. Nucleic Acids Res. 21, 5890-5895.

Vanhouten, B., and Sancar, A. (1987). Repair of n-methyl-n'-nitro-n-nitrosoguanidine-induced DNA damage by ABC excinuclease. Journal of Bacteriology 169, 540-545.

VanHouten, B., and Snowden, A. (1993). Mechanism of action of the *E. coli* UvrABC nuclease: Clues to the damage recognition problem. Bioessays 15, 51-59.

Vanhouten, B., Gamper, H., Holbrook, S. R., Hearst, J. E., and Sancar, A. (1986). Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position. Proc. Natl. Acad. Sci. USA 83, 8077-8081.

Verhage, R. A. (1996). Transcription-coupled and global genome repair in *Saccharomyces cerevisiae* [Ph.D. Dissertation]. (University of Leiden, Leiden).

Verhage, R., Zeeman, A. M., Degroot, N., Gleig, F., Bang, D. D., Vandeputte, P., and Brouwer, J. (1994). The *RAD7* and *RAD16* genes, which are essential for pyrimidine dimer removal from the silent mating-type loci, are also required for repair of the nontranscribed strand of an active gene in *S.cerevisiae*. Mol. Cell. Biol. 14, 6135-6142.

Verhage, R. A., van Gool, A. J., de Groot, N., Hoeijmakers, J. H. J., van de Putte, P., and Brouwer, J. (1996a). Double mutants of *Saccharomyces cerevisiae* with alterations in global genome and transcription-coupled repair. Mol. Cell. Biol. 16, 496-502.

Verhage, R. A., Zeeman, A., -M., Lombaerts, M., van de Putte, P., and Brouwer, J. (1996b). Analysis of gene-and strand-specific repair in the moderately UV-sensitive *Saccharomyces cerevisiae rad23* mutant. Mutation Research 362, 155-165.

Verhage, R. A., Van der Putte, P. and Brouwer, J., (1996c). Repair of rDNA in *S.cerevisiae*: *RAD14*-independent strand specific NER of RNA polymerase I transcribed genes. Nucleic Acid Research. 24, 1020-1025

Verhage, R. A., Heyn, J., vandePutte, P., and Brouwer, J. (1997). Transcription elongation factor S-II is not required for transcription-coupled repair in yeast. Mol. Gen. Genet. 254, 284-290.

Vermeulen, W., Jaeken, J., Jaspers, N. G. J., Bootsma, D., and Hoeijmakers, J. H. J. (1993). Xeroderma-pigmentosum complementation group-G associated with cockayne-syndrome. American Journal of Human Genetics 53, 185-192.

Vrieling, H., vanZeeland, A. A., and Mullenders, L. H. F. (1998). Transcription coupled repair and its impact on mutagenesis. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 400(1-2 Sisi), 135-142.

Wallace, S. S. (1994). DNA damages processed by base excision repair: Biological consequences. International Journal of Radiation Biology 66, 579-589.

Wang, C.-I., and Taylor, J.-S. (1991). Site specific effect of thymine dimer formation on dAn.dTn tract bending and its biological implications. Proc. Natl. Acad. Sci. USA 88, 9072-9076.

Wang, Z. G., Svejstrup, J. Q., Feaver, W. J., Wu, X. H., Kornberg, R. D., and Friedberg, E. C. (1994). Transcription factor-b (TFIIH) is required during nucleotide-excision repair in yeast. Nature 368, 74-76.

Ward, J. F. (1988a). DNA damage produced by ionizing-radiation in mammalian-cells - identities, mechanisms of formation, and reparability. Progress in Nucleic Acid Research and Molecular Biology 35, 95-125.

Ward, J. F. (1988b). The chemical basis of radiation biology. Nature 331, 128.

Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A., Weiner, A.M., (1988), Molecular biology of the gene, 4th edition.

Weber, C. A., Salazar, E. P., Stewart, S. A., and Thompson, L. H. (1990). *ERCC2*: cDNAs cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast *RAD3*. EMBO J. 9, 1437-1447.

Weeda, G., Vanham, R. C. A., Masurel, R., Westerveld, A., Odijk, H., Dewit, J., Bootsma, D., Vandereb, A. J., and Hoeijmakers, J. H. J. (1990a). Molecular-cloning and biological characterization of the human excision repair gene ERCC-3. Mol. Cell. Biol. 10, 2570-2581.

Weeda, G., Vanham, R. C. A., Vermeulen, W., Bootsma, D., Vandereb, A. J., and Hoeijmakers, J. H. J. (1990b). A presumed DNA helicase encoded by ERCC-3 is involved in the human repair disorders xeroderma-pigmentosum and cockayne syndrome. Cell 62, 777-791.

Wei, H., and Frenkel, K. (1991). *In vivo* formation of oxidized DNA bases in tumor promoter-treated mouse skin. Cancer Research 51, 4443-4449.

Wei, Y. H. (1998). Oxidative stress and mitochondrial DNA mutations in human aging. Proceedings of the Society for Experimental Biology and Medicine 217, 53-63.

Weiss, B., Cunningham, R. P., Chan, E., and Tsaneva, I. R. (1988). AP endonucleases of *Escherichia coli*. In Mechanisms and consequences of DNA damage processing, E. C. Friedberg, and P. C. Hanawalt, eds. (New York: Alan R. Liss, Inc.), pp. 133-142.

Williamson, M. S., Game, J. C., and Fogel, S. (1985). Meiotic gene conversion mutants in *Saccharomyces cerevisiae*. 1. Isolation and characterisation of PMS1-1 and PMS1-2. Genetics 110, 609-646.

Wood, M. L., Dizdaroglu, M., Gajewski, E., and Essigmann, J. M. (1990). Mechanistic studies of ionizing radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in viral genome. Biochemistry 29, 7024-7032.

Woods, C. G. (1998). DNA repair disorders. Archives of Disease in Childhood 78, 178-184.

You, H. J., Swanson, R. L., and Doetsch, P. W. (1998). *Saccharomyces cerevisiae* possesses two functional homologues of *Escherichia coli* endonuclease III. Biochemistry 37, 6033-6040.

You, Z. Y., Feaver, W. J., and Friedberg, E. C. (1998). Yeast RNA polymerase II transcription *in vitro* is inhibited in the presence of nucleotide excision repair: Complementation of inhibition by holo-TFIIH and requirement for Rad26. Mol. Cell. Biol. 18, 2668-2676.

Zawel, L., Kumar, K. P., and Reinberg, D. (1995). Recycling of the general transcription factors during RNA polymerase II transcription. Genes Dev. 9, 1479-1490.

Acknowledgement

I am deeply grateful to my wonderful supervisor professor Ray Waters for introducing me to the fascinating world of DNA damage and Repair and providing me with the opportunity and inspiration to do a research in this subject. I would like to thank him for his excellent supervision during the course of this project, his encouragement, support and his patience in constructive discussions and reading my thesis. I also would like to thank Mrs Eileen Waters for her kindness and hospitality.

I had the great pleasure of being part of a marvellous research group. I owe many sincere thanks to Dr Andy Scott, Dr Valerie Meniel, and Dr Yumin Teng for their expert instructions help and advice. I am deeply grateful to Sue Fieldings and Andrea Walley for their great help at the start of my work. I would like to thank all the people mentioned above and the rest of our research team, past and present members: Prapaypat Klungsupya, Nerys Morse, Shirong Yu, Margaret Clathworthy, Cath Gallagher, Greg Mc Donough, Yachuan Yu, Dr Jose Antonio Ferreiro, Dr Shisheng Li, Dr Ned Powell, Dr Simon Reed and Dr Gary Jones for their help, friendship, and encouragement.

Many thanks goes to Professor David Skibinsky for his advice in statistical analysis of my data. I also take this opportunity to say thanks to the other academic members. Dr Richard Tilney Bassett, Dr Helgi Öpik, Professor Jim Parry, Dr Liz Parry, Dr Milton, Dr Nigel Jones, Professor Jon Beardmore, Dr Paul Dyson, Dr Hugh Jones, Professor Norman Ratcliff, Professor Andrew Rowley, Dr Mick Fordy, Dr Steve Wainwright, Mr Roger Elias, Professor Merrett, Dr Kevin Flyn, that I had the honour of being their student when I was doing my first degree. I profoundly feel indebted to all these great people.

Lots of thanks goes to Sally James and Karen Sykes; to Margaret Morris, Dot Morris, Liz Carrick and Mr Walmsley; to Ann Hopkins and Mark Roberts; and to the staff of the natural science and the main library for their very kind help and support.

I had a chance maybe of a life time to meet so many lovely people from so many different parts of the world during the course of my PhD: Nasima Ali, Dr Zulficar Hassan, Mehmood Kayany, Dr Ayse Boybek, Morad Diklitash, Dr Errol Eroglu, Dr Gulsum Gunes, Gulnur, Dr Adele Kim, Dr Ayse Al-obaidli, Dr Morteza Hashemzadeh Chaleshtory, Dr Farhad Amini, Dr Sohrab Rezvani, Dr Mohammed Purkazemi, Mingguo Tnag, Dr Hameed Baseri, Meisa Alwohhaib, Bina, Lawra, Dr Enaam Marafi, Dr Hakan Ulukan and Mrs Aysegul Ulukan, Elias, Kat Cheriton, Margaret Linley, Hawazen Yousef, Paola Mignosi, Haleh, David and little Nima, Dr Aarabi and Minu jun, Dr Ali and Mrs Ali, Mrs Conway, Tim and Rhiannan, Gwen, Faranak, Suzan Allcock, Michel Fleet, Kereshme, Bob Randig, Erick Roderick, Rob Williams, Andy Pitman, Mairwyn Ewans, Derek Thomas, Dr Rupet Lewis, Dr Bryan Mayers, Dr Thierry Hermine, Dr Hamberto Quesada, Gareth Lavery, Dr Hiko, Dr Roz Anna Sueiro Benavides, Chiara Corso, Honglin Song and Zheng Ye, Di Elwell, Ena Jones, Jeen Leamon, Chris Beynon, Dr Gareth Jenkins, Ryan Guest, Justine Williamson, Jon Strefford, Dr Helen Lake, Dr Joanne Fenton, Dr Paul Lewis, Dr Paul Herron, Dr Steve Conlan, Dr Nimal Balanatram, Dr Steve McKain.

I would like to say special thanks to Dr Tunjar Demir for precious privileges given so genuinely; for kindness, for understanding, for loads of encouragement.

When I came to Swansea I knew nobody. But then among the names mentioned so far in my acknowledgement or names that are inadvertently missed (or miswritten), there were people who gave their valuable time to supervise and teach me, show me the techniques, answer my questions, share and discuss ideas. All those with so much patience and kindness. There were even people who I didn't get a chance to know much but their little help in the lab, their friendly faces and smiles meant a great deal of support, or those who I only came to know through their interesting

and inspiring talks in research seminars. There were people through whom I learnt a lot about traditions, customs, religions, ideas, societies and countries. There were people with whom I shared so much fun and enjoyed so many tea and lunch breaks, parties, cycling and walking trips. There were people together we shared a house. There were people who became my soul mates. There were of course people I am pleased to meet recently. I learnt a lot from you all. The support and encouragement in any way that I received from each one of you during this time was vital when it was most needed. This time certainly wouldn't be as beautiful without you. I am greatly deeply thankful to you all. I will miss you.

And my love goes to my family *specially maman, baba and my dearest grandmother, To Naser and Zari; To Hadi and to the rest of my extended family too numerous to mention in here (Their name is written in the acknowledgement of my heart). Thank you for your endless support and looking after me so well, for your patience, the life giving strength and happiness you gave me, your great hospitality when I was coming home, for your love, care, support and encouragement. Thanks for being close to my heart no matter how far the distances between us. Thanks for everything. This work would not be achieved without you. I love you all.*

My love and thanks to the very loved ones who are sadly missed but never forgotten. How delightful I would be to still have you at this stage of my life. I hope that you are also sharing my happiness at the completion of this work wherever you are.

I also would like to thank the Iran ministry of culture and higher education for their support and providing the opportunities to make this work possible and the office of national and expatriate Iranian scholars and scientists for their encouragement and keeping me in touch with home scientists and conferences.